PROJECT TITLE

STRUCTURAL ANALYSIS OF VARIOUS STARCH DEBRANCHING ENZYMES AND THEIR PRODUCTION IN *BACILLUS* SPECIES

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WAKNAGHAT

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This is to certify that the work entitled "**STRUCTURAL ANALYSIS OF VARIOUS STARCH DEBRANCHING ENZYME AND THEIR PRODUCTION IN BACILLUS SPECIES"** pursued by **Shubham Vashishtha** (123802) & **Tushar Singh Barwal** (123811) in partial fulfillment for the award of degree Bachelor of Technology in Biotechnology from Jaypee University of Information Technology, Wakhnaghat has been carried out under my supervision. This part of work has not been submitted partially or wholly to any other University or Institute for the award of any degree or appreciation.

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOLS	ABBREVIATIONS
LB	Luria Broth
LMRS	Lactobacillus de Man, Rogosa and Sharpe
DNS	3,5-Dinitrosalicylic acid
dH_2O	Distilled water
OD	Optical density
DNA	Deoxyribonucleic Acid
pH	Potential of hydrogen
TNE	Tris Sodium(Na) EDTA
SET	Sodium (SDS)EDTA Tris
SDS	Sodium dodecyl sulphate
EDTA	Ethylenediaminetetraacetic Acid
TE	Tris EDTA
EtBr	Ethidium Bromide
NCDC	National Collection Of Dairy Cultures
MTCC	Microbial Type Culture Collection and Gene Bank
°C	Degree Celsius
g	Gram
µl	Microliter
µg	Microgram
ml	Milliliter
mg	Milligram
rpm	Rotation per minute
%	Percentage
BSA	Bovine Serum Albumin
NCBI	National center for Biotechnology information
BLAST	Basic Local alignment Search Tool
PDB	Protein Data Base
FASTA	FAST Alignment

CHAPTER 1

Review of literature

1.1. INTRODUCTION

Enzymes are highly instrumental to the starch industry, and there journey so far is highly successful. Pullulanase (debranching enzyme) is such an enzyme, which in the recent years have emerged as an outstanding participant in the processing of different forms of starch. Pullulanse exclusively hydrolyse the α-1, 6 glycosidic bonds this helps the enzyme to be used in wide applications across various industries. The enzyme has incredibly elevated the efficiency of the starch saccharification into simple sugars which could be used directly or could be further fermented to many other useful products. Lots of research has been done till date, touching various aspects of pullulanase comprising of its production, purification, characterization, genetic engineering and structural studies. The current paper is an effort to put forward the concise form of all such aspects, creating an insight into the efficient starch converter; pullulanase.

The use of enzyme is not new and dates back to the ancient Greeks. All enzymes are proteins with an exception of certain RNA molecules and have diverse functions. In the cellular system the enzymes perform varied functions varying from the maintenance of the cell to the defence of cell against pathogens .Over the past few decades, use of enzymes as a biocatalyst have tremendously contributed to the diverse branches of industries. Starch industry is one such consumer whose demands for higher and higher quantity is gaining momentum with need for specialised modified starch products.

Starch contains glucosidic bond which remains highly stable under alkaline condition but on treatment with an acid or an enzyme break into constituent glucose molecule. Starch being a polymer of very high molecular weight is composed of

1) Amylose a linear chain molecule (alpha 1, 4 linkages)

2) Amylopectin a branch polymer (alpha 1, 6linkage) of glucose.

Enzymes like amylase, neopullulanase, isopullulanase are capable of cleaving the alpha 1, 4 linkages in the starch and enzymes like pullulanase and alpha amylase are able to hydrolyse the alpha 1, 6 linkages in the starch. The only difference lies in the fact that pullulanase can hydrolyse the alpha 1, 6 linkages in pullulan and amylopectin whereas with the alpha amylase can hydrolyse only alpha 1, 6 bond in amylopectin and glycogen.

Pullulanase (Pullulan 6-glucanohydrolase [EC 3.2.1.41] is a debranching enzyme that has recently established its great importance in starch conversion. It belongs to alpha amylase family also known as glycosyl hydrolase family 13 [6]. It breaks the alpha 1, 6 glycosidic bonds in pullulan, producing maltotriose and other polysaccharides like starch, producing oligosaccharides. In addition, we have pullulan hydrolase type I (neopullulanase) and type II (isopullulanase) which are only capable of cleaving alpha 1, 4 glycosidic linkages in pullulan. Conversion of starch into useful products requires breakage into oligosaccharides which is achieved at a very high temperature within 3 steps: gelatinization, liquefaction and saccharification. Traditional starch converting enzymes -alpha amylase, beta amylase have limitation of not being able to hydrolyse alpha-1,6 glucosidic bonds forming alpha and beta limit dextrins respectively [3,4] whereas glucoamylase acts slowly and reverts dextrose into isomaltose decreasing glucose production [3]. To overcome the barriers faced by other enzymes, pullulanase is used in saccharification process in combination with glucoamylase or alpha amylase for starch conversion [22]. Research studies are still on the way to discover the thermo stable pullulanase that can be proved as an efficient starch converting enzyme on a commercial scale. Due to the capability of efficient hydrolysis of starch, pullulanase have grabbed wide area of industrial sector under their applications. Alkaline pullulanase can be used in detergents, dishwashing [20, 54]. It helps in the high-maltose corn syrup and high-fructose corn syrup production which are used in pharmaceutical industries and food industries [55, 14]. In starch processing industry pullulanase is used in combination with other amylolytic enzymes to enhance the efficiency of saccharification process and produce high yield of sugars [52]. In the comparative studies pullulanase has shown a higher rate of hydrolysis when compared with enzymes like isoamylase over certain substrate.

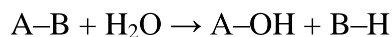
Substrate	Relative rate of Hydrolysis	
	Isoamylase	Pullulanase
Pullulan	Very low	100
Pure amylopectin	100	15
Glycogen	124	1
Rabbit liver	111	0.6

Table 1-Action of pullulanase and isoamylase on various substrates

Now a day's different industries are craving for extremophilic enzymes; thermophilic enzymes being in the hit list as they can easily survive the harsh industrial processes. The temperature optimum for these thermostable pullulanase is ranged between 850 ^{0}C to 1050 ^{0}C without the need of any Ca^{2+} ions and absence of any substrate. That is why in recent years in recent years, much of the research is done on pullulanase from thermophilic microorganisms [7, 13].

1.2. TYPES OF HYDROLYTIC ENZYMES

In biochemistry an enzyme is said to be hydrolytic enzyme if it is capable of hydrolysis of the chemical bond. The general representation for such a reaction is given as-



$$A\text{–}B + H_2O \rightarrow A\text{–}OH + B\text{–}H$$

Starch is composed of two types of bonds that are alpha 1-4 and 1-6 linkage so need a significant amount of treatment with a wide variety of enzymes before we can use the glucose. Industrially alpha amylase is the enzyme which is extensively used, along with alpha amylase there are also some other enzymes like Pullulanases and pullulanase hydrolase whose application and demand is increasing as more study is being done in their respective fields.

Type	EC number	Bonds Processed	Preferred substrate	End products	References
Pullulanase type I	3.2.1.41	Alpha-(1-6)	Oligo-and Polysaccharides, pullulan	Trimer (maltotriose)	[5]
Pullulanase type II (amylopullulanase)	3.2.1.41	Alpha-(1-6) and Alpha-(1-4)	Pullulan, Poly-and oligosaccharides and oligosaccharides (starch)	Trimer (maltotriose) Mixture of glucose, maltose, and maltotriose	[45]
Pullulan hydrolase type I (neopullulanase)	3.2.1.135	Alpha-(1-4)	Pullulan	Panose	[5]
Pullulan hydrolase type II (isopullulanase)	3.2.1.57	Alpha-(1-4)	Pullulan	Isopanose	[7]

Pullulanase Hydrolase Type III	3.2.1-	Alpha-(1-6) and Alpha-(1-4)	Pullulan Starch, amylose and amylopectin	Mixture of Panose, maltose and maltriose	[24]
Beta amylase	3..2.1.2	Alpha (1-4)	Starch and glycogen	maltose	[8]
Iso-amylase	3.2.1.68	Alpha (1-6)	Glycogen and amylopectin	Maltose and maltriose	[49]
Alpha amylase	3.2.1.1	Alpha (1-4)	Starch and glycogen	Glucose and maltose	[43]

Table 2- List of major starch hydrolysing enzymes

1.3. MECHANISM OF ACTION OF PULLULANASE

Industrially important starch is highly complex substrate and contains approximately 80% of amylopectin. The branching point occurs on an average after every 20 to 25 D-glucose units, this states that there are on an average 4% to 5% of (alpha-1-6 glycosidic linkages) in the starch. Various starch substrates have varying ratios of amylose and amylopectin. Depending on the ratio of the amylose and amylopectin the treatment varies.

Starch	**Type**	**Size range (micro-meter)**	**Shape**	**Amylose (%)**	**Amylopectin (%)**
Sago	Pith	5-35	Oval, truncated	27	73
Corn	Cereal	3-36	Round polygonal	28	72
Potato	Tuber	5-100	Oval, Spherical	21	79

Wheat	Cereal	2-35	Round reticular	28	72
Tapioca	Root	4-35	Oval, truncated	17	83
Rice	cereal	3-8	Polygonal ,angular	17	83

Table 3- Composition of major starch substrate used in industries.

The alpha 1-6 linkage present in the various starch molecule acts as a barrier for the action of other hydrolytic enzymes during saccharification. Unlike the alpha amylase which bypasses the branching point the enzyme pullulanase is able to break this linkage hence increasing the saccharification.

Industries tend to minimise the reaction as it increments the cost and the final glucose yield in industrial process. The yield in the saccharification reaction can be improved by using enzymes like the pullulanse and amylase in tandem; in this the pullulanse will act on the 1-6 residues and the amylose on the 1-4 residues. The use of a debranching enzyme will increase rate of overall saccharification and reduce the amount of the amylase and time required in the reaction. Reduction up to 60% in the amount of amylase has been reported so far.

Sources:

The pioneer work of isolation of pullulanase was done in 1961 by Bender and Wallenfels, from mesophillic bacteria *Klebsiella pneumonia*. By far diverse number of sources has been marked for the presence of pullulanase; comprising of plants, bacteria and fungi. Plant sources comprise of rice endosperm, spinach, millet. Also evidences for fungal Pullulanases such as from *Hypocrea jacorina (QM9414*) and bacterial pullulanase are there. A large number of thermostable pullulanses have been isolated from different moderately thermophilic, thermophilic and hyperthermophillic species of bacteria and archaea, for eg. moderately thermophilic bacteria *Bacillus acidopullulolyticus* and *Bacillus flavocaldarius* KP, the thermophilic bacteria *Thermus aquaticus* YT-1, *Thermus caldophilus* GK- and *Bacillus thermoleovorans* and the extreme anaerobic thermophilic *bacteria Caldocellulosiruptor saccharolyticus, Fervidobacterium pennivorans* and *Thermotoga maritime*.

1.4. PULLULANASE SERVING VARIOUS INDUSTRIES

With the increasing demands of processed starch, the applications of starch debranching enzymes are also increasing considerably.

1. **Starch industry:**

   Pullulanase by making use of its exclusive hydrolyzing capability cleaves the amylopectin portion of starch and leaves behind high content of amylose portion resulting in the production of high amylose starch. This starch could be used directly in adhesive products or could be further processed leading to the formation of resistant starch which is very healthy for colon of the large intestine.

2. **Food industry:**

   Since pullulanase is used in combination with amylolytic enzymes in the saccharification process, so it aids in the production of glucose, fructose and maltose by the conversion of starch. Now a day's high maltose and fructose corn syrups are very much in demand for they are serving as excellent low caloric and high quality sweeteners in various food products.

3. **Baking industry:**

   Baking industry is one another industry that heavily relies on starch modifying enzymes. The major problem faced by the industry is maintenance of the quality of the baked products by prevention of staling. Research studies have revealed that staling is the consequence of certain undesirable physical and chemical changes in the baked food stuff. Physical changes comprise of loss of moisture content, loss of crispness of the crust, changes in flavor and increase in firmness of the crumb. Pullulanase are being used in this context to reduce the stickiness in the bread, increase the loaf volume and shelf life of the baked product. Chemical changes constitute crystallization of the starch content (mainly amylopectin) present in the baked product due to starch retro gradation or reorganization into an unstructured or gelatinized form. This change initiates when baked products undergo cooling and progresses towards aging causing rigidity in the baked food. Studies show that if there is better degradation of amylopectin (by making use of amylases), the retro gradation of starch will be hindered and thus staling could be prevented. Thus, pullulanase can enhance the anti-staling

efficiency as it leads to the efficient hydrolysis of amylopectin fraction of the starch in the baked food.

4. **Pharmaceutical industry:**

The enzyme aids in the production of pure maltose syrup and glucose syrups. Maltose is intravenously injected into diabetic patients instead of glucose. So the pure maltose production by the enzyme contributes to pharmaceutical industry. Also pullulanase enhances the production of cyclodextrins which are used as complexing materials in pharmaceuticals.

5. **Detergent industry:**

Pullulanase is used in laundry detergents as an additive for it acts on the branched portion of the starchy stain and efficiently helps alpha amylase in removing dirt. Mainly alkaline pullulanase and amylo-pullulanase are preferred to be used in the detergents.

6. **Brewing industry:**

Pullulanase assure the complete hydrolysis of the alpha 1-6 glucosidic bonds of the starchy material used in beer production and thus leads to maximum fermentation of the wort by the action of yeast. This is because if the starch will be completely hydrolyzed into simple sugars, then the yeast will have ample food to act upon and hence carry higher fermentation of the wort in return. Reduced amount of dextrins in the beer not only leads to improved quality but also a low calorie beer which is good for health.

1.5. PRODUCTION OF PULLULANASE:

Recent studies evident that lot of work has been done on the production of pullulanase, both by the native method i.e. fermentation as well as by genetic engineering. Concise information regarding the research work on both the production methods has been discussed ahead in this review.

Fermentation studies on pullulanase:

After the optimization of different parameters on the pilot scale, the production of enzyme is then carried on large scale in high volume fermenters. Fermentation can be either submerged type or

solid state fermentation. Mostly submerged fermentation is preferred from industrial point of view as in this type of fermentation the monitoring and control of the different environmental parameters is comparatively easier. On average most of the research studies have revealed dependence on submerged fermentation for the enzyme production [44, 47]. However, due to some advantages like low capital investment, simple technique, low energy requirement, solid state fermentation (SSF) has also been a method of choice. There are evidences where pullulanase production has been carried using solid state fermentation as well [40].

Submerged fermentation for pullulanase production has been carried out by various scientists. In 2009, Siew Ling Hii and his coworkers made an effort for the production of pullulanase from *Raoultella planticola DSM2* 4617 using submerged type of fermentation. They optimized the type of carbon and nitrogen sources to be used as well as concluded the optimum temperature and pH conditions for the enzyme production. They found sago starch to be the best carbon source as it gave highest extracellular enzyme production (182Upul g/starch) and maximum activity (0.95 U/ml) as compared to potato (0.85 U/ml), tapioca (0.68 U/ml) and corn (0.14 U/ml). Different optimization studies have evidenced starch to be the best carbon source for the pullulanase production both in submerged as well as solid state fermentation; however starch has been used in different forms. Soluble starch resulted in maximum pullulanase production when SM Noorwez et al, 2006 carried submerged fermentation using *Geobacillus thermoleovorans* with an incubation time of 20 hrs. Similar results were found in the same year by Subhash.U. Nair and group while conducting solid state fermentation of about 12 hrs. The production of enzyme from the organism *Bacillus cereus*, another study that supported the use of soluble starch as a good carbon source for pullulanase production was done by Mohamed. A and friends using *Bacillus licheniformis*, When solid state fermentation was carried out with the same bacteria, results were in favor of corn bran + rice bran as the carbon source [25]. In 2000, P.M reddy with his coworkers carried out solid state fermentation with *Clostridium thermosulfurogens SV2* as the producer organism at 600C for 1 day and analyzed the best carbon source. Using different strain of *Clostridium thermosulfurogenes (SVM17)* for the amylopullulanase production and got best results with raw sago starch as the carbon source. In the following year i.e. 2011, when S. Mridula and group practiced the submerged fermentation with the same bacteria, they found maltose to be the best carbon source. So in nutshell, choice of carbon sources varied according to

different microorganisms used and the type of fermentation opted. Peptone was best suited when analyzed from nitrogen studies.

1.6. BIOCHEMICAL CHARACTERIZATION

1. pH:

pH is an important parameter that greatly affects the activity of the enzyme. Pullulanases are believed to show their activity within a broad pH range from 2- 10 (acidic to alkaline). The optimum pH range for pullulanase activity lies within a range of 5.5 to 6.0. Research studies on enzymes like *Clostridium thermohydrosulfuricum*, *Bacillus cereus*, *Thermococcus kodakarensis* KOD1 (19) verify this optimum range of pH. Optimum pH of 4.5 (acidic range) has been shown by *Fervidobacterium pennivorans*. Pullulanases of alkaline range have also been reported, *Bacillus halodurans* with pH 10, *Anoxybacillus* with pH 7, *Anaerobranca gottschalkii* with pH 8. There are some results in which there is drift in the optimum pH range of the enzyme activity; the pH of *Clostridium thermohydrosulfuricum* shifted towards the acidic range (i.e 5.6 changed to 5.2) when temperature was lowered below 60^0C.

2. Temperature:

Like pH, temperature is also very important parameter that affects the growth and the production of the extracellular enzyme. Thermophilic microorganisms show highest activity between temperature range 55-80^0C and some hyperthermophilic microorganisms show highest activity produced at 80 – 115^0C. The optimum activity of pullulanase lies within the range of 55- 100^0C. *Anoxybacillus*, *Bacillus halodurans*, *Fervidobacterium pennivorans* showed their optimal activity at moderate temperatures 55^0C, 50^0C and 60^0C respectively. While pullulanses with very high optimum temperatures were reported from *Anaerobranca gottschalkii* with 70^0C, *Clostridium thermohydrosulfuricum* with 85^0C and *Thermotoga neapolitana*. *Thermococcus kodakarensis* KOD had optimum temperature at extreme 100^0C.

3. Metal ions:

Most of the metal ions have inhibitory effect on the activity of pullulanase. Metal ions like Ni, Co, Cd, Mg, Cu, Zn , Na are the inhibitors, as in *Bacillus cereus* .Though, they may show slight or strong inhibitory effect varying within pullulanses from different microorganisms, whereas Ca ions mostly have stimulating effect on the pullulanse activity, as in *Bacillus halodurans* . Ca ion showed strong stimulatory effect on pullulanase from *Bacillus cereus*

such that it increased the activity by 170%. Hg ions also have remarkable inhibitory effect on enzyme activity as in *Thermotoga neapolitana.*

4. Substrate specificity:

Pullulanase has efficient specificity for the different substrates; pullulan is the major one followed by amylopectin, starch, dextrins, glycogen, amylose and soluble starch. The enzyme produced by *Anoxybacillus* showed specificity towards pullulan and starch and hydrolyzed them to end products maltotriose and glucose respectively. Pullulanase from *Thermotoga neapolitana* was capable of hydrolyzing pullulan, amylopectin, starch and glycogen but had no effect on amylose, indicating itself as true pullulanase (pullulanase I).

5. Chemical reagents:

Different chemical reagents EDTA, SDS, N-bromosuccinimide (NBS), beta mercaptoethanol etc. have inhibitory, stimulatory or no effect on the activity of the enzyme, varying according to the producer microorganism of the enzyme and the type of the enzyme. Like EDTA enhanced the activity of pullulanase from *Thermus thermophilus* while it had no effect on the activity of the enzyme produced by *Geobacillus thermoleovorans* US 105 and *Bacillus* sp. AN-7. N-bromosucinimide (NBS) mostly causes complete inactivation of the enzyme [*Thermotoga neapolitana*, Pyrococcus woesei].

6. General features, common to alpha amylase family:

Pullulanase belongs to 13 glycosyl hydrolases family (alpha amylase family) and thus shows hydrolysis activity, capable of hydrolyzing the alpha glucosidic bonds (alpha-1-4 and alpha1-6).There is presence of four conserved regions in the catalytic site. Some of the conserved regions in the primary sequence may also be involved in the stability of the barrel structure containing catalytic residues. Domains A and C have similarity to the other enzymes of the alpha amylase family. The (β/α) 8 or TIM barrel structure containing the catalytic site residues present in pullulanase is also common among the enzymes of the alpha amylase family.

Organism	Optimal Temp.	Optimal pH.	Molecular weight	Stimulating metal ions	Inhibiting metal ions	reference
Thermus thermophiles	70	5.5	52	Mn^{2+}, Fe^{2+}	Hg^{2+},Cu^{2+}	[60]
Bacillus sp. AN-7	90	6	106	Ca^{2+}, Mn^{2+}	Hg^{2+}	[28]
Thermotoga neapolitana	80	5-7	93	Mg^{2+}, Ca^{2+}	Cu^{2+}, Hg^{2+}, Zn^{2+}, Ni^{2+},	[24]
Pyrococcus woesei	100	6	90	Ca^{2+}	Ni^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Cr^{2}	[51]
Geobacillus thermoleovoran	70	6	160	Ca^{2+}	No inhibitory effect	[63]
Anoxybacillus	60	7.5	225	K+, Fe2+, Fe3+,	No inhibitory effect	[11]
Bacillus halodurans	50	10	37	Ca^{2+}	Zn^{2+}, Cu^{2+}	[5]
Bacillus cereus H1.5	55	6	93	Ca^{2+}	Ni^{2+}, Co^{2+}, Cd^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+},	[43]
Anaerobranca gottschalkii	70	8	96	No Stimulating Effect	No inhibitory effect	[6]
Clostridium thermohydrosulf	85-90	5.6	-	Ca^{2+}	Zn^{2+}, Cu^{2+}	[22]
Fervidobacterium	80	6	43	No Stimulating Effect	Zn^{2+}, Cu^{2+}, Fe^{2+}	[7]
Thermotoga maritima M3B8	90	6	-	Li^{2+}, Na^{2+}, K^{+}, Ca^{2+}, Mg^{2+}	Co^{2+}, Al^{3+}, Zn^{2+}, Mn^{2+},	[8]
Desulfurococcus	85	5	132	No Stimulating Effect	Zn^{2+}, Cu^{2+}, Fe^{2+}	[1]
Lactococcus lactis	45	4.5	73.9	Co^{2+}	Hg^{2+}	[59]

Table4- Biochemical properties

1.7. CRYSTAL STRUCTURE DESCRIPTION:

The enzyme is composed of total 5 domains: N1, N2, N3, A and C. A is the catalytic domain in the structure of pullulanase. N1, N2 and N3 represent the N-terminal of the enzyme while C domain represents the C-terminal of the enzyme. The N-terminal and C-terminal domains are composed of a motif of two-sheeted b-sandwich structures each composed of 5b/3b, 4b/4b, 3b/4b, and 5b/3b, respectively, while the (b/a)8-barrel structure is present in the catalytic A domain. Hydrogen bonds and Vander wall interactions keep the N2, N3, A and C domains tightly bounded. N1 domain has the carbohydrate binding site and calcium binding site as well. Exceptionally in Group B *Streptococcus* Pullulanase Crystal Structure, the NI domain containing carbohydrate binding molecule was lacking but it retained the catalytic activity toward alpha glucan substrates [17]. Total 5 calcium sites are present in the structure of pullulanase, one in the N1 domain, three in the A domain and one in the C domain as depicted in the figure [39].

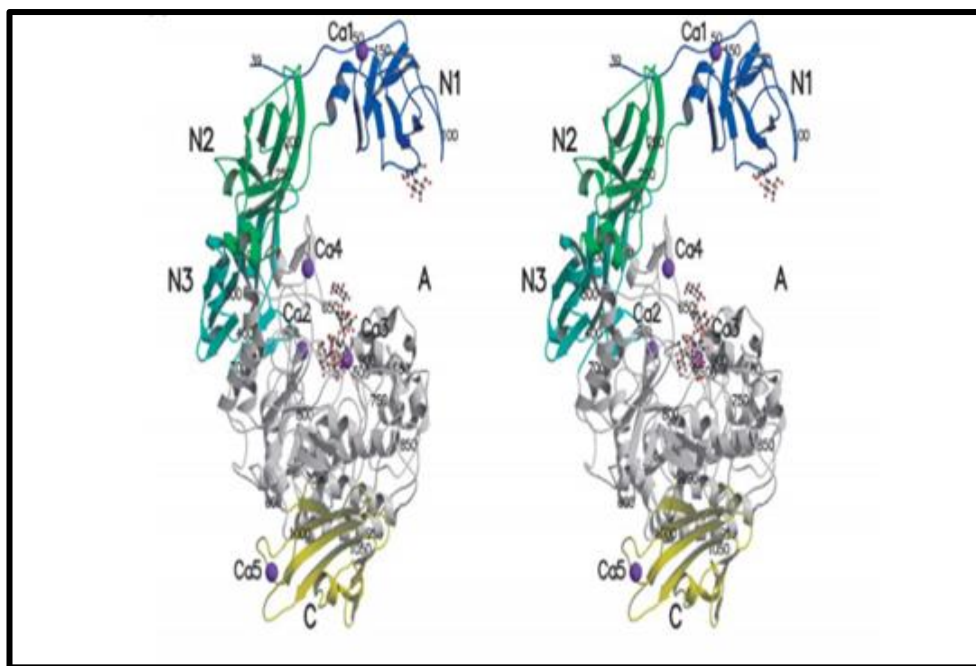


Figure 1: Overall structure of the pullulanase with G4 (ribbon stereo diagram). The Figure shows N1 (blue), N2 (green), N3 (cyan), A (white) and C (yellow) domains, together with five calcium ions (purple sphere) and two G4 (ball and-stick) in the active site [39].

Future prospects:

Pullulanase because of its wide area of applications, demands much more research studies and emphasis, so that it could be better understood in each and every aspect of its characteristic features. This will help in its more exploration, thereby increasing its industrial applicability and also uplift its economic and commercial value. Starch is the basic raw material in number of industries and so is the demand of the starch hydrolyzing enzymes. The exclusive hydrolyzing capacity of pullulanase is of great importance and should not be neglected as the enzyme has bright future prospects. Furthermore the Availability of large o of highly thermotolerant variants of pullulanase provide us with the capability to use the enzyme at an higher temperature's which is very important for the starch industry.

1.8. ORGANISMS USED DURING STUDY

	Bacillus subtilis	***Bacillus subtilis***	***Lactobacillus plantarum***	***Bacillus subtilis***
MTCC No.	121	1790	2941	NCDC 71
Strain designation	3610	QB1133	20205	NCIMB 8054
Growth medium	Luria broth	Luria broth	MRS medium	Luria broth
Temperature	30^0C	37^0C	30^0C	30^0C
Growth condition	Aerobic	Aerobic	Aerobic	Aerobic
Incubation	24 hrs.	24 hrs.	48 hrs.	24 hrs.
Sub-culturing period	30 days	30 days	30 days	30 days
Reference	MTCC	MTCC	MTCC	NCDC

Table 4- List of organism used

1.9. Rational of the project

Most enzymes used in the industry are plagued by constrains like inability to work at high temperature, need for co-factors and inability to work over wide variety of substrates. Pullulanse is a potential answer to all these problems faced by other chemicals. Further more industries tend to minimise the reaction as it increments the cost and the final glucose yield in industrial process. The yield in the saccharification reaction can be improved by using enzymes like the pullulanse and amylase in tandem; in this the pullulanse will act on the 1-6 residues and the amylose on the 1-4 residues. The use of a debranching enzyme will increase rate of overall saccharification and reduce the amount of the amylase and time required in the reaction. Reduction up to 60% in the amount of amylase has been reported so far. Most enzyme production these days is being carried out with the help of fungal sources our study tends to explore the potential of bacterial sources for such an operation.

Objectives of the project-

- Analysis of activity of various starch debranching enzymes from *Bacillus* sources.
- Optimization and production of starch debranching enzymes from *Bacillus sp*.
- Comparative studies of active sites of various starch debranching enzyme by sequence and structural analysis.
- Multiple sequence alignment.
- 3D Structural alignment and comparison of various active domains responsible for the enzyme activity.

CHAPTER 2

MATERIALS AND METHODS

2.1. REVIVAL OF GLYCEROL STOCKS

- LB and LMRS media (HI Media) was prepared and poured in test tubes for autoclaving in duplets for *Bacillus subtilis* (121, NCDC71, 1790) and *Lactobacillus plantarum (2941),* autoclaved at 121^0 C at 15 psi for 15 mins.
- Bacterial cultures maintained as glycerol stocks at -80^0 were collected from the cell repository.
- Cultures were kept on ice for an hour to bring them to 4^0 C and then kept at room temperature for culturing. (*Note: cultures were not directly brought to room temperature in order to prevent heat shock and damage to cell lines*)
- Tubes of LB and LMRS media were inoculated with 10 µl of glycerol stock for respective organism.
- Cultured test tubes were incubated at 37^0 C at 140 RPM for 24 hours and 48 hours for *Bacillus* and *Lactobacillus* respectively.
- Tubes were observed after incubation.

a. TO CHECK CONTAMINATION IN THE REVIVED CULTURES

- LB and LMRS agar media were prepared with 1.5 % agar (Merck) concentration in duplets and autoclaved.
- Autoclaved media was poured in 6 plates of LB and 2 plates of LMRS media and kept under UV sterilization for 30 mins to solidify.
- Plates after solidification were kept at 37^0 C for 36 hours in incubator to check contamination if any.
- From the seed cultures prepared above small amount of inoculum was taken using sterile inoculation loop.
- Simple and quadrant streaking was done on the plates from each sample respectively.
- Plates after inoculation were incubated at 37^0 C for 24 and 36 hours for *Bacillus* and *Lactobacillus* respectively.
- Results were observed and noted after incubation.

b. SUBCULTURE OF THE REVIVED COLONIES AND PREPARARTION OF GLYCEROL STOCKS FOR LONG TERM STORAGE

- After contamination check both LB and LMRS media was prepared for subculture and growth of the organism.
- At the same time 30% glycerol was made and autoclaved.
- 100 ml of LB and LMRS broth was made for each organism and 10 ml broth in test tubes was prepared for sub culturing and seed cultures, autoclave.
- From the above prepared plates small amount of inoculum was taken using sterile inoculation loop and test tubes were inoculated and incubated for 24 hours and 36 hours respectively.
- After growth in seed culture media 500 µl of culture was taken and pipetted in 1.5ml Eppendorf and equal volume of glycerol (30%) was added and sealed using parafilm and stored at -80^0 C.
- Also for subculture 100 µl of inoculum was taken and flasks were inoculated for 24 hours and 36 hours respectively.

2.2. QUALITATIVE ANALYSIS OF AMYLASE ACTIVITY BY STARCH IODINE TEST

Media used: Starch agar media

Theory: The starch agar test is done to check for the production of amylase enzyme by the organism. The iodine reacts with starch to form a dark blue-colored complex. Clear area around the growth of the culture after the addition of iodine indicates the breakdown of starch by Amylase.

Protocol [55]:

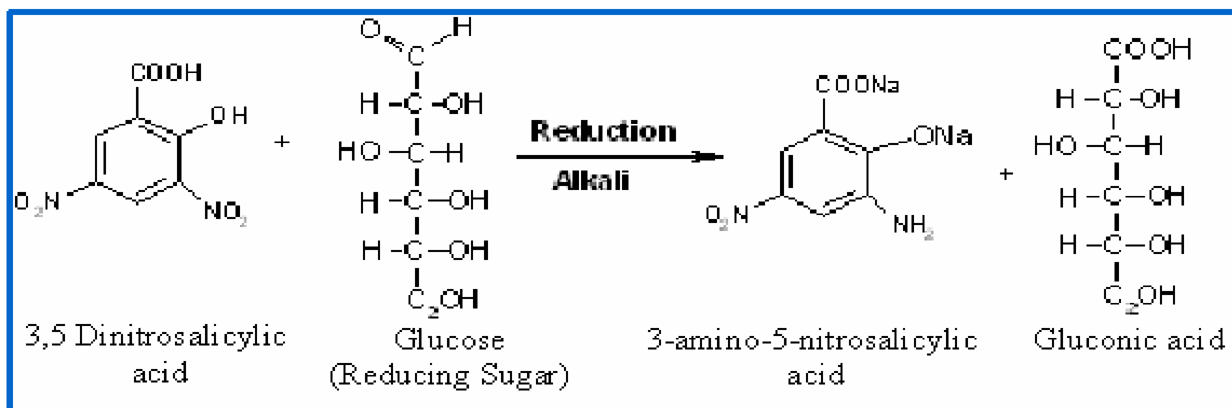
- Using the above composition the starch agar media was made and autoclaved, media was poured in plates accordingly for duplets.
- Plates were inoculated with cultures of *Bacillus* and *Lactobacillus* and simple streaking was done.

- Plates were incubated at 37^0 C for 24 hours and 36 hours respectively.
- After growth plates were flooded with gram iodine and observed.

2.3. QUANTITATIVE ANALYSIS OF ENZYME ACTIVITY BY DNS METHOD

Chemicals used: DNS reagent, Starch solution *(1% starch solution prepared in 100 ml 0.02 M Sodium phosphate buffer pH 7),* Buffer (*0.02 M sodium phosphate buffer pH 7 with 0.006 M sodium chloride),* 1mg/ml Glucose solution *(0.1 g Glucose in 100 ml of* dH_2O*).*

Theory: This tests for the presence of free carbonyl group (C=O), the reducing sugars. It involves the oxidation of the aldehyde functional and ketone functional group. Also, 3, 5-dinitrosalicylic acid (DNS) is reduced to 3-amino-5-nitrosalicylic acid under alkaline conditions, as shown below:



Standard curve for glucose:

- Components were added as shown in the table (1) and incubated respectively.

Table 1						
Tube no.	**Glucose Concentration (µg/ml)**	**Glucose Volume (ml)**	**dH_2O (ml)**	**DNS reagent (ml)**	**Heat in Boiling water bath for 5 mins And cool them**	**dH_2O (ml)**
1	**0**	**0**	**1**	**2**		**7**
2	**200**	**0.2**	**0.8**	**2**		**7**
3	**400**	**0.4**	**0.6**	**2**		**7**
4	**600**	**0.6**	**0.4**	**2**		**7**
5	**800**	**0.8**	**0.2**	**2**		**7**
6	**1000**	**1**	**0**	**2**		**7**

- After completion 200 µl of the sample was loaded in the ELISA plate wells and OD was taken at 540 nm in ELISA plate reader.

Test for enzyme activity:

- Culture was grown for organism in LB and LMRS media for 24 and 36 hrs. Respectively in duplets.
- Cultures were collected in 10 ml each sterile Tarson tubes and centrifuged at 2000 rpm for 8 mins at 4^0 C, Supernatant was collected in new Tarson 15 ml tubes and kept on ice.
- Test tubes were taken and marked accordingly.
- Both controls were taken Enzyme control (*enzyme + buffer*) and substrate control (*substrate + buffer*), and incubated for 5 mins at 30^0 C.
- Further proceeded as per the table (2).

Table 2

Tube no.	Enzyme (ml)		Substrate (ml)	Buffer (ml)		DNS (ml)		dH_2O (ml)
Cs	0.0	Incubate for 3 mins at 30^0 C	0.5	0.5	Incubate for 5 mins at 30^0 C	2	Heat in boiling water for 5 mins	7
1_c	0.5		0.0	0.5		2		7
11	0.5		0.5	0.0		2		7
12	0.5		0.5	0.0		2		7
2_c	0.5		0.0	0.5		2		7
21	0.5		0.5	0.0		2		7
22	0.5		0.5	0.0		2		7
3_c	0.5		0.0	0.5		2		7
31	0.5		0.5	0.0		2		7
32	0.5		0.5	0.0		2		7
4_c	0.5		0.0	0.5		2		7
41	0.5		0.5	0.0		2		7
42	0.5		0.5	0.0		2		7

(**Note:** Marking goes as Cs- substrate control

1,2,3,4 are codes for organisms (121, 1790, NCDC 71, 2941 respectively)

Subscript c donates controls for respective organisms

Second digit represents the sample number of that organism, like 11 is the first sample of 121)

- Samples were cooled and OD taken at 540 nm.

Test for Enzyme activity at varying temperatures:

- Test tubes were washed and marked, further processing done as per the table (3).

Table 3

Tube no.	Enzyme (ml)	Incubate for 3 mins at 25, 30 , 40, 50, 60^0 C for each set of samples individually	Substrate (ml)	Buffer (ml)	Incubate for 5 mins at 25, 30 , 40, 50, 60^0 C for each set of samples individually	DNS (ml)	Heat in boiling water for 5 mins	dH_2O
Cs	0.0		0.5	0.5		2		7
1_c	0.5		0.0	0.5		2		7
11	0.5		0.5	0.0		2		7
12	0.5		0.5	0.0		2		7
2_c	0.5		0.0	0.5		2		7
21	0.5		0.5	0.0		2		7
22	0.5		0.5	0.0		2		7
3_c	0.5		0.0	0.5		2		7
31	0.5		0.5	0.0		2		7
32	0.5		0.5	0.0		2		7
4_c	0.5		0.0	0.5		2		7
41	0.5		0.5	0.0		2		7
42	0.5		0.5	0.0		2		7

- Samples were cooled and OD taken at 540 nm.

(**Note:** Marking goes as Cs- substrate control

1,2,3,4 are codes for organisms (121, 1790, NCDC 71, 2941 respectively)

Subscript c donates controls for respective organisms

Second digit represents the sample number of that organism, like 11 is the first sample of 121)

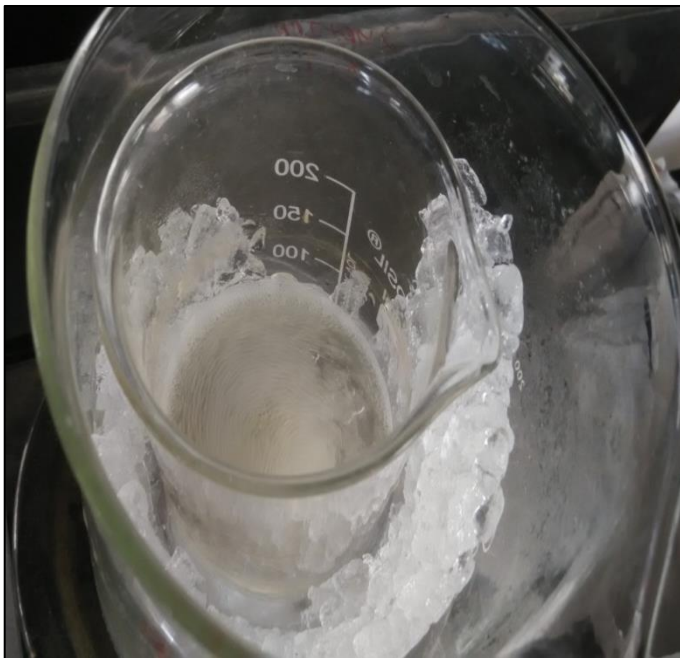
2.4. PROTEIN PURIFICATION BY AMMONIUM PRECIPITATION AND ACTIVITY ASSAY

Theory: [54] Many cytosolic proteins are water soluble and their solubility is a function of the ionic strength and pH of the solution. The commonly used salt for this purpose is Ammonium Sulphate, due to its high solubility even at lower temperatures. Proteins in aqueous solutions are heavily hydrated, and with the addition of salt, the water molecules become more attracted to the salt than to the protein due to the higher charge. This competition for hydration is usually more favorable towards the salt, which leads to interaction between the proteins, resulting in aggregation and finally precipitation. The precipitate can then be collected by centrifugation and the protein pellet is re-dissolved in a low salt buffer.

Since different proteins have distinct characteristics, it is often the case that they precipitate (or ‘salt out’) at a particular concentration of salt.

Protocols:

- Cultures of the selected organisms with good enzymatic activity were taken NCDC 71 and 2941 and grown in LB and LMRS media respectively in duplets.
- Cultures were collected in 50ml tarson tubes and centrifuged at 4000 rpm for 10 mins and supernatant was collected in new tarson in duplets.
- From the table of ammonium sulfate precipitation for 0-30 % cut amount of ammonium sulfate was calculated.
- 8.2 g of ammonium sulfate each was slowly added pinch by pinch to the medium
- containing protein and was continuously stirred at 4^0 C



Picture (1): Setup for Ammonium sulphate

- Ammonium sulphate was mixed completely and the media was kept at 4^0 C for overnight precipitation.
- Next day sample was centrifuged at 4000 rpm for 20 mins, supernetant was taken and collected in a new tarson tube and pellet was resuspended in sodium phosphate buffer and assay was done with the DNS reagent.
- AS per in the table, and OD were taken at 540 nm.

Table 4								
Tube no.	**Enzyme (ml)**	**Incubate for 3 mins at 30^0 C**	**Substrate (ml)**	**Buffer (ml)**	**Incubate for 5 mins at 30^0 C**	**DNS (ml)**	**Heat in boiling water for 5 mins**	**dH_2O (ml)**
Cs	**0.0**		**0.5**	**0.5**		**2**		**7**
311_c	**0.5**		**0.5**	**0.0**		**2**		**7**
311	**0.5**		**0.0**	**0.5**		**2**		**7**
411_c	**0.5**		**0.5**	**0.0**		**2**		**7**
411	**0.5**		**0.5**	**0.0**		**2**		**7**

(**Note:** Marking goes as Cs- substrate control

1,2,3,4 are codes for organisms (121, 1790, NCDC 71, 2941 respectively)

Subscript c donates controls for respective organisms

Second digit represents the sample number of that organism, like 11 is the first sample of 121

The third digit represents the cut i.e. 0-30% is 1 30-60% is 2 and 60-90% is 3)

- Now for the second cut of 30-60% amount of ammonium sulphate was calculated from the table.
- 9.05 g of ammonium sulphate each was slowly added pinch by pinch to the medium containing protein and was continuously stirred at 4^0 C
- Ammonium sulphate was mixed completely and the sample was kept at 4^0 C for overnight precipitation.
- Next day sample was centrifuged at 4000 rpm for 20 mins, supernetant was taken and collected in a new tarson tube and pellet was resuspended in sodium phosphate buffer and assay was done with the DNS reagent following the same protocol as above.

- Now for the third cut of 60-90% amount of ammonium sulphate was calculated from the table.
- 10.05 g of ammonium sulphate each was slowly added pinch by pinch to the medium containing protein and was continuously stirred at 4^0 C
- Ammonium sulphate was mixed completely and the sample was kept at 4^0 C for overnight precipitation.
- Next day sample was centrifuged at 4000 rpm for 20 mins, supernetant was taken and collected in a new tarson tube and pellet was resuspended in sodium phosphate buffer and assay was done with the DNS reagent following the same protocol as above.
- Similarly the above experiment was performed with the other set of samples and OD was taken for all the samples at 540nm.

2.5. QUALITATIVE ANALYSIS OF PULLULANASE ACTIVITY BY PULLULAN DEGRADING TEST

a. Test 1 with Pullulan agar media composition 1(annexure 9)

Media used: Pullulan agar media

Theory: Pullulan is a polysaccharide polymer consisting of maltotriose units, also known as α-1, 4 α-1, 6-glucan. Pullulanase produced by bacteria degrades pullulan at α-1,4 linkage and both 1,4 and 1,6 in amylopullulanase to give glucose monomer thus making media colorless from the area where it degrades the substrate making the test positive.

Protocols:

- Seed culture was prepared for all four strains 121, 1790, NCDC 71, 2941 in LB and
- LMRS media respectively in 10 ml test tubes.
- 250 ml of Pullulan agar media was made and autoclaved, media was poured in plates in duplets.
- Plates were inoculated with cultures of *Bacillus* and *Lactobacillus* and simple streaking was done.
- Plates were incubated at 37^0 C for 24 hours and 36 hours respectively.

b. Test 2 with Pullulan agar media composition 2(annexure 10)

Media used: Pullulan agar media

Protocols:

- Seed culture was prepared for all four strains 121, 1790, NCDC 71, 2941 in LB and
- LMRS media respectively in 10 ml test tubes.
- 250 ml of Pullulan agar media was made and autoclaved, media was poured in plates in duplets.
- Plates were inoculated with cultures of *bacillus* and *lactobacillus* and simple streaking was done.
- Plates were incubated at 37^0 C for 24 hours and 36 hours respectively.

2.6. QUANTITATIVE ANALYSIS OF PULLULANASE ACTIVITY BY DNS METHOD

Chemicals used: DNS reagent, Pullulan solution *(1% starch solution prepared in 100 ml 0.02 M Sodium phosphate buffer pH 7),* Buffer (*0.02 M sodium phosphate buffer pH 7 with 0.006 M sodium chloride),* 1mg/ml Glucose solution *(0.1 g Glucose in 100 ml of* dH_2O*)*

Theory: The main principle is same for the DNS test explained in experiment 2.3. Here pullulan is used as a substrate for the enzyme pullulanase as pullulan is a maltotriose composed of glucose monomer. When pullulanase acted on the substrate pullulan it converted it into monomers of glucose which were detected using DNS reagent at 540 nm.

Standard curve for glucose:

- Components were added as shown in the table (5) and incubated respectively.

Table 5

Tube no.	Glucose Concentration (µg/ml)	Glucose Volume (ml)	dH_2O (ml)	DNS reagent (ml)	Heat in Boiling water bath for 5 mins And cool them	dH_2O (ml)
1	0	0	1	2		7
2	200	0.2	0.8	2		7
3	400	0.4	0.6	2		7
4	600	0.6	0.4	2		7
5	800	0.8	0.2	2		7
6	1000	1	0	2		7

- After completion 200µl of the sample loaded in the ELISA plate wells and OD was taken at 540 nm in ELISA plate reader.

a. Activity assay at temperature 50^0C (Abdulla, A. A. (2014).)

- Culture was grown for organism in LB and LMRS media for 24 hrs. in duplets.
- Cultures were collected in 10 ml each sterile Tarson tubes and centrifuged at 2000 rpm for 8 mins at 4^0 C, Supernatant was collected in new Tarson 15 ml tubes and kept on ice.
- Test tubes were taken and marked accordingly.
- Both controls were taken Enzyme control (*enzyme + buffer*) and substrate control (*substrate + buffer*), and incubated for 20 mins at 50^0 C.
- Further proceed as per table (6).

Table 6							
Tube no.	**Enzyme (ml)**	**Substrate (ml)**	**Buffer (ml)**		**DNS (ml)**		**dH_2O**
Cs	0.0	1	0.5	Incubate for 20 mins at 50^0 C	2	Heat in boiling water for 5 mins	6.5
1_c	0.5	0	1		2		6.5
11	0.5	1	0		2		6.5
12	0.5	1	0		2		6.5
2_c	0.5	0	1		2		6.5
21	0.5	1	0		2		6.5
22	0.5	1	0		2		6.5
3_c	0.5	0	1		2		6.5
31	0.5	1	0		2		6.5
32	0.5	1	0		2		6.5
4_c	0.5	0	1		2		6.5
41	0.5	1	0		2		6.5
42	0.5	1	0		2		6.5

(**Note:** Marking goes as Cs- substrate control

1,2,3,4 are codes for organisms (121, 1790, NCDC 71, 2941 respectively)

Subscript c donates controls for respective organisms

Second digit represents the sample number of that organism, like 11 is the first sample of 121)

- Samples were cooled and OD was taken at 540 nm.

b. Activity assay after Ammonium precipitation

- Cultures of the organisms were taken and grown in LB and LMRS media respectively in duplets.
- Cultures were collected in 50ml tarson tubes and centrifuged at 4000 rpm for 10 mins and supernatant was collected in new tarson in duplets.
- From the table of ammonium sulfate precipitation for 0-60 % cut amount and 0-90% (2941) cut amount of ammonium sulfate was calculated.
- Ammonium precipitation was done following the same protocol as in 2.5.

- Protein collected after precipitation was used for activity analysis following the same table as above.
- Samples were cooled and OD taken at 540 nm.

2.7. PROTEIN ESTIMATION USING BRADFORD METHOD

Chemicals used: Bradford reagent, BSA standard solution (1 mg/ml)

Theory: The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change.

a. Standard curve for Bradford using BSA:

- Components were added as shown in the table (7) and incubated respectively.

Table 7

Tube no.	BSA Concentration (µg/ml)	BSA Volume (µl)	dH_2O (µl)	Amount of sample added to microtiter plate in triplet(µl)	Bradford reagent (µl)	
1	0	0	30	10	2	Let it rest for 5 mins and take OD at 595 nm.
2	200	6	24	10	2	
3	400	12	18	10	2	
4	600	18	12	10	2	
5	800	24	6	10	2	
6	1000	30	0	10	2	

- After completion 200µl of the sample loaded in the ELISA plate wells and OD was taken at 595 nm in ELISA plate reader.

b. Protein estimation for unknown samples:

- Cultures of the organisms were taken and grown in LB and LMRS media respectively.
- 2ml of culture was collected in Eppendorf from each sample and was centrifuged at 4000 rpm for 10 mins and supernatant was collected in new Eppendorf in triplets.
- With respective controls of samples experiment was performed
- Further proceeded as per the table (8).

Table 8

Tube no.	Unknown sample(µl)	LB/LMRS Media(µl)	Bradford reagent(µl)	
1c	0	10	200	Let it rest for 5 mins and take OD at 595 nm.
11	10	0	200	
12	10	0	200	
2c	0	10	200	
21	10	0	200	
22	10	0	200	
3c	0	10	200	
31	10	0	200	
32	10	0	200	
4c	0	10	200	
41	10	0	200	
42	10	0	200	

(**Note:** Marking goes as Cs- substrate control

1,2,3,4 are codes for organisms (121, 1790, NCDC 71, 2941 respectively)

Subscript c donates controls for respective organisms

Second digit represents the sample number of that organism, like 11 is the first sample of 121).

- OD was taken at 595 nm in ELISA plate.

2.8. COMPARATIVE ACTIVITY ANALYSIS OF BOTH ENZYMES AMYLASE AND PULLULANASE

Protocols: Activity assay of both the enzymes were done using DNS method as follows:

- Cultures of the organisms were taken and grown in LB and LMRS media respectively in duplets.
- 2 ml of sample was taken in Eppendorf's and centrifuged at 8000 rpm for 2 mins at 4^0C and supernatant was collected in separate Eppendorf's for further analysis.

a. Amylase activity

- Test tubes were taken and marked accordingly.
- Both controls were taken Enzyme control (enzyme + buffer) and substrate control (substrate + buffer), and incubated for 5 mins at 30^0 C.
- Further proceed as per the table (9). Substrate used in 1% starch.

Table 9

Tube no.	Enzyme (ml)		Substrate (ml)	Buffer (ml)		DNS (ml)		dH_2O
Cs	0.0	Incubate for 3 mins at 30^0 C	0.5	0.5	Incubate for 5 mins at 30^0 C	2	Heat in boiling water for 5 mins	7
1_c	0.5		0.0	0.5		2		7
11	0.5		0.5	0.0		2		7
12	0.5		0.5	0.0		2		7
2_c	0.5		0.0	0.5		2		7
21	0.5		0.5	0.0		2		7
22	0.5		0.5	0.0		2		7
3_c	0.5		0.0	0.5		2		7
31	0.5		0.5	0.0		2		7
32	0.5		0.5	0.0		2		7
4_c	0.5		0.0	0.5		2		7
41	0.5		0.5	0.0		2		7
42	0.5		0.5	0.0		2		7

(**Note:** Marking goes as Cs- substrate control

1,2,3,4 are codes for organisms (121, 1790, NCDC 71, 2941 respectively)

Subscript c donates controls for respective organisms

Second digit represents the sample number of that organism, like 11 is the first sample of 121)

- Samples were cooled and OD taken at 540 nm.

b. Pullulanase activity

- Test tubes were taken and marked accordingly.
- Both controls were taken Enzyme control (enzyme + buffer) and substrate control (substrate + buffer), and incubated for 20 mins at 50^0 C.
- Further proceed as per the table (10). Substrate used is 1% pullulan.

Table 10

Tube no.	Enzyme (ml)		Substrate (ml)	Buffer (ml)		DNS (ml)		dH_2O
Cs	0.0	Incubate for 3 mins at 30^0 C	0.5	0.5	Incubate for 10 mins at 30^0 C	2	Heat in boiling water for 5 mins	7
1_c	0.5		0.0	0.5		2		7
11	0.5		0.5	0.0		2		7
12	0.5		0.5	0.0		2		7
2_c	0.5		0.0	0.5		2		7
21	0.5		0.5	0.0		2		7
22	0.5		0.5	0.0		2		7
3_c	0.5		0.0	0.5		2		7
31	0.5		0.5	0.0		2		7
32	0.5		0.5	0.0		2		7
4_c	0.5		0.0	0.5		2		7
41	0.5		0.5	0.0		2		7
42	0.5		0.5	0.0		2		7

(**Note:** Marking goes as Cs- substrate control

1,2,3,4 are codes for organisms (121, 1790, NCDC 71, 2941 respectively)

Subscript c donates controls for respective organisms

Second digit represents the sample number of that organism, like 11 is the first sample of 121)

- Samples were cooled and OD taken at 540 nm.
- Enzyme concentrations taken for both the samples were greater than 20µg/ml.

2.9. DNA ISOLATION

Reagents and Chemicals used: TNE buffer, 70% Ethanol, SET buffer, 1 vol. PCI, 3 M Sodium acetate, Absolute ethanol, T.E buffer.

Theory: The role of various components in DNA isolation protocol is as follows:

- **The extraction buffer (SET Buffer)**: Consist of a detergent such as SDS which disrupts the membranes, a chelating agent such as EDTA which chelates the magnesium ions required for DNase activity, a buffer which is Tris HCl at pH 8 and a salt such as sodium chloride (*Component of TNE buffer)* which result in precipitation by neutralizing the negative charges on the DNA so that the molecules can come together.
- **Phenol chloroform extraction:** DNA solutions commonly contain undesirable contaminants that are chiefly made of proteins. Method of purifying them is phenol –chloroform extraction by which the nucleic acid solution is extracted by a volume of phenol: chloroform: Isoamyl alcohol (1: 25: 24). Centrifugation is performed and the upper aqueous phase is transferred to a new tube while avoiding the interphase. The contaminants are denatured and accumulate in the organic phase or in the marginal layer between the two phases and the nucleic acids are preserved in the aqueous phase.
- **Precipitation of nucleic acids**: Alcohol precipitation is the most commonly used method for nucleic acid precipitation. This requires diluting the nucleic acid with a monovalent salt i.e. successively washing with Sodium acetate that removes Cellular and Histone proteins bound to DNA and adding alcohol to it and mixing gently. The nucleic acid precipitated spontaneously and can be pelleted by centrifugation. The salts and alcohol remnants are removed by washing with 70% alcohol.
- **Re-suspending DNA**: The nucleic acid pellet can be re-suspended in either sterile distilled water or TE buffer for storage.

Protocol:

- Organisms were grown beforehand in their specific sterile media and collected in 15 ml Tarson tubes and centrifuged at 4000 rpm for 10 mins.

- Pellet of each sample was collected and resuspended in 900µl TNE buffer and was centrifuged at 15000 rpm for 5 mins.
- Pellet was resuspended in 70% ethanol (ice cold) and was placed on ice for 20mins. Suspension was centrifuged at 12000 rpm for 5 mins.
- Pellet was resuspended in 480µl of SET buffer; tubes were kept at -20^0 C for 20 mins and immediately transferred to water bath at 68^0 C for 10 mins.
- 1 volume of PCI (1: 24: 25) was added and mixed gently by inversion, centrifugation was done at 5000 rpm for 10 mins and upper phase was collected and placed in new tubes.
- 100µl of 3 M Sodium acetate and equal volume of absolute ethanol (-20^0 C) was added to each tube and mixed gently; centrifugation was done at 5000 rpm for 10 mins.
- Pellet was washed with 70% ethanol at 5000 rpm for 10 mins
- Pellet resuspended in 100µl TE Buffer and stored at 4^0 C.

VISUALIZATION OF DNA BY GEL ELECTROPHORESIS AND DETECTION BY SPECTROPHOTOMETRY

Gel electrophoresis: DNA being negatively charged move towards the positively charged side and is visualized by mixing gel with EtBr that intercalate in between the base pairs and make it appear as orange colored in UV light. SB buffer here provides the free ions for the movement of DNA and agarose is a matrix, whose porosity can be changed depending upon the concentration and thereby separation depending upon the size of sample. Loading Dye is used to keep a track on DNA and to make it settle in the wells while loading.

- Gel was prepared using SB buffer (Annexure 7), 0.5 g of agarose was added in 50ml of SB buffer and heated till the agarose dissolves (1% gel).
- Gel was cooled down till 45-55^0 C and then 2.5µl EtBr was added to the gel (*Note: since hot gel will denature the EtBr).*
- Gel was poured slowly in the casting tray with comb on and was left for solidification.
- After solidification of gel comb was removed slowly and gel apparatus was flooded with SB buffer till it covered the surface and the gel got dipped.

- Sampled were mixed with the loading dye (3: 1) respectively and loaded into the wells.
- Gel was run for 60 mins at 100 V voltages.
- Gel after run was slowly removed from the apparatus and visualized under Gel doc system at 302/312-nm.

Nanodrop spectrophotometer: It is a device used to detect the DNA molecule using wavelength or light. It depends upon the beer lambert's law and DNA is detected under 260 nm wave length.

- Nanodrop plate was loaded with 1µl of reference that is the TE buffer in which the DNA was resuspended.
- DNA from different samples was loaded with 1µl of volume on the plate.
- Reading was taken using spectrophotometer at 260 nm, 260/280 nm for purity.

2.10. Acquiring Protein sequence and structure.

Databases used: NCBI Protein database (collection of protein from various sources).

Protein BLAST using PDB database (Protein BLAST is an alignment tool used to find the similar and identical sequences that matches the Entered query, using PDB database you can find the structure similar to the sequence of the protein.

Phyre^2: Phyre^2 is a service for predicting the 3-dimensional structure of a protein amino acid sequence.

Protocols:

- NCBI was used to get the protein sequences of the selected enzymes in *Bacillus* species.
- www.ncbi.nlm.nih.gov was opened.
- Search query for individual enzymes was entered in the search box and searched.
- Most relevant sequences were selected.
- Sequence files were downloaded in FASTA format.

- Sequences were used and standard protein BLAST was done to find the most relevant protein structure.
- Sequences with good identity and e value were selected and downloaded from PDB.
- For the sequences whose protein structure was unavailable an online database tool for structure prediction was used (phyre2 http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).
- Query sequence was entered to predict the structure.
- Predicted structure of the sequence was downloaded.

2.11. Multiple sequence alignment using Clustalw

Database used: Clustalw (Online multiple sequence alignment tool)

Protocols:

- The above mentioned sequences were aligned using Clustalw (http://www.ebi.ac.uk/Tools/msa/clustalo/).
- Protein sequence in FASTA format were entered in the query box and aligned.

2.12. Structure alignment of the Proteins

a. Alignment of whole protein structure:

Tool used: Pymol (It is a molecular visualization system, which can be used for structural alignment and visualization)

Protocols:

- Protein structures were visualized using Pymol.
- Structures were preset to publication mode and provided with single color tag.
- Alignment of the structures was done and studied.

b. Domain analysis

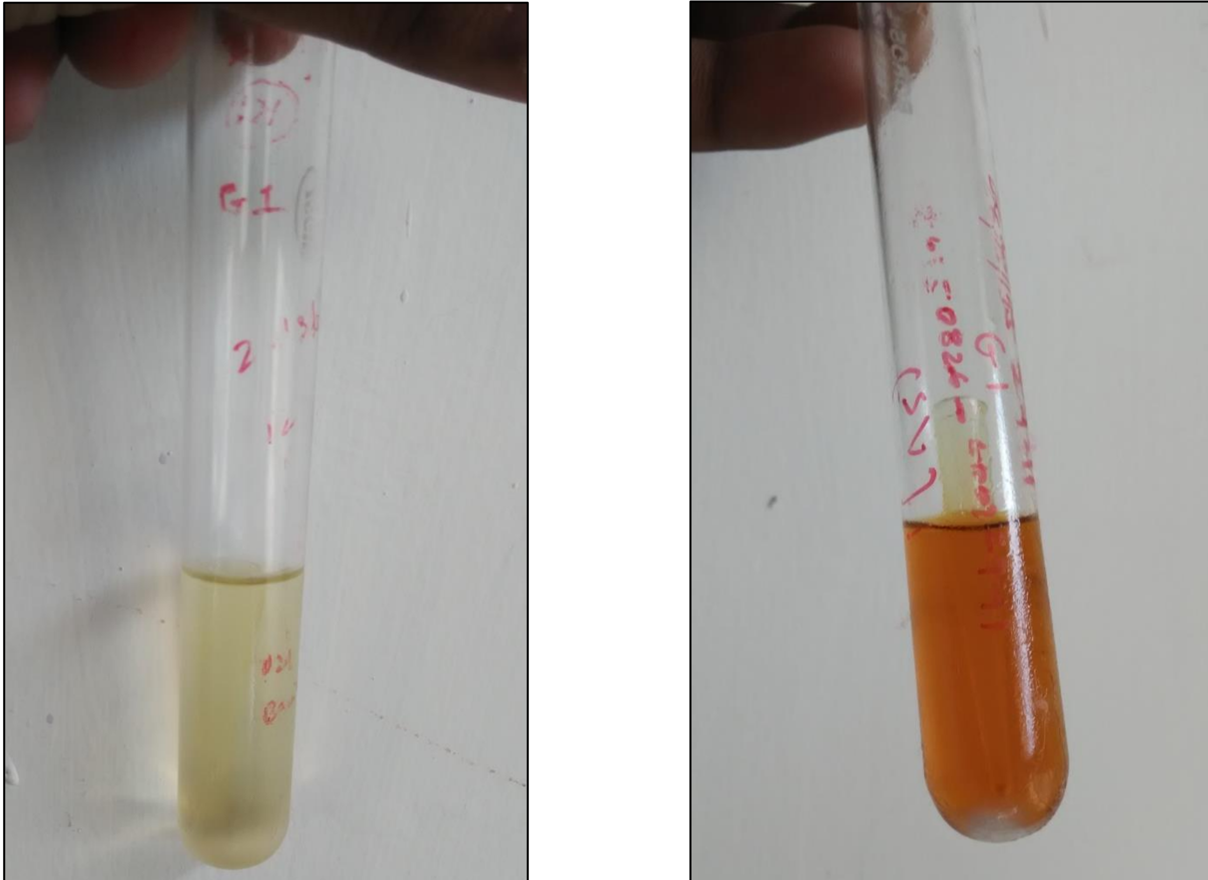
- Domains present in the enzyme were analyzed and studied.

CHAPTER 3

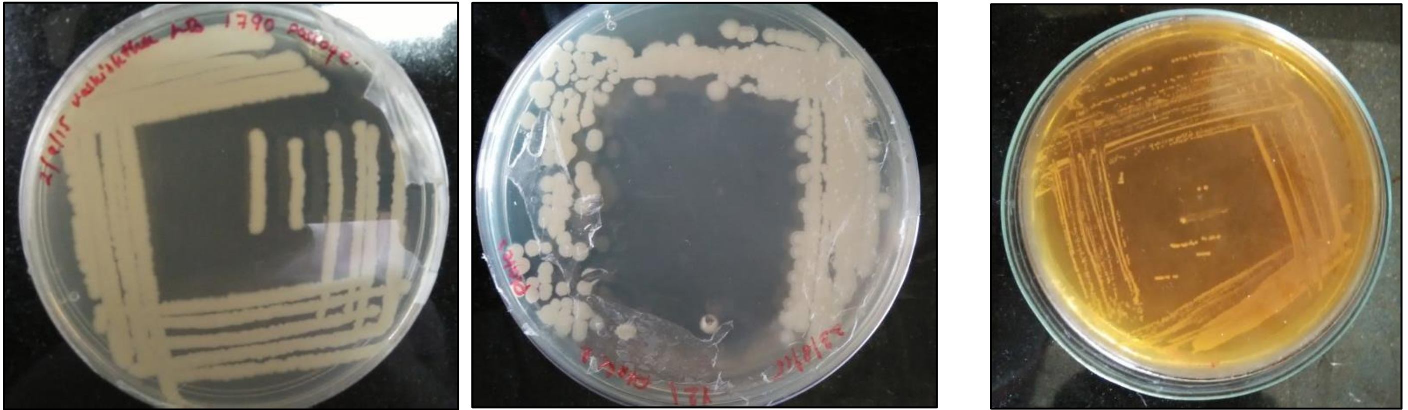
RESULTS

3.1. REVIVAL OF GLYCEROL STOCKS, CHECK FOR CONTAMINATION AND SUBCULTURING.

The cultures were revived all there strains of bacillus 1790, NCDC 71, 121 and lactobacillus 2941 without any contamination and their G1 generation was grown from the isolated colonies that we got after 2-3 sub culturing and cross checking for their contamination.



Pic1. Revival of glycerol stocks

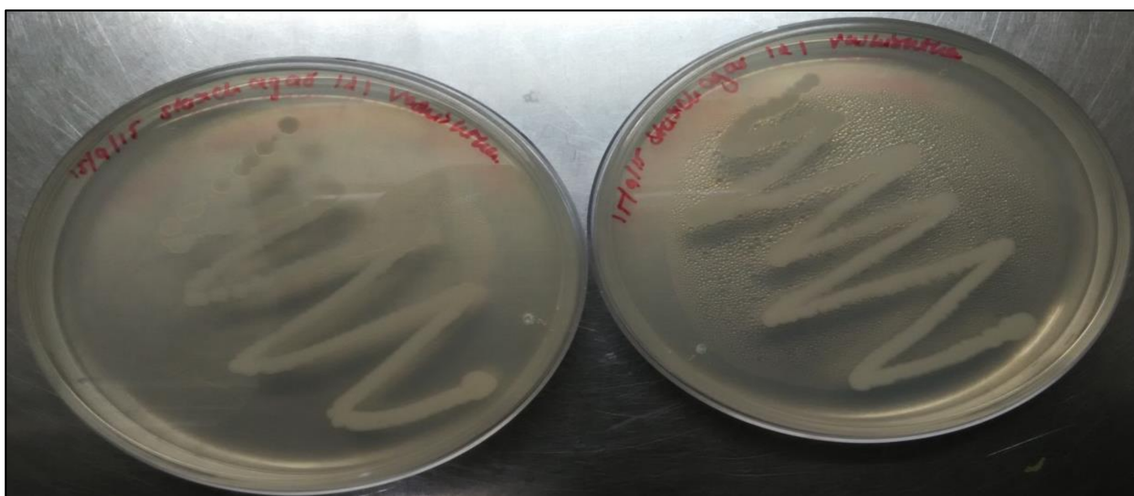


Pic2. Plates of cultures revived cultures checked for contamination

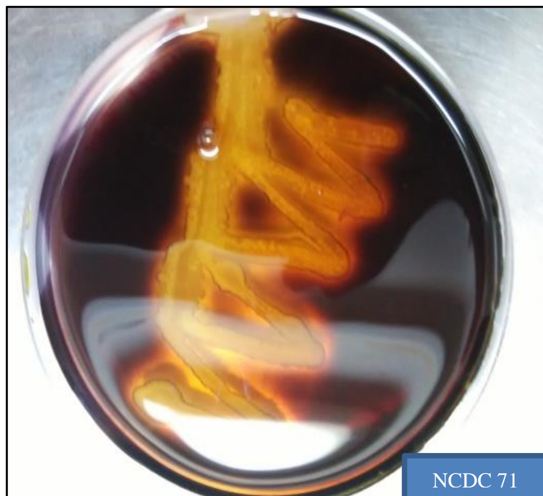
3.2. QUALITATIVE ANALYSIS OF AMYLASE ACTIVITY BY STARCH IODINE TEST

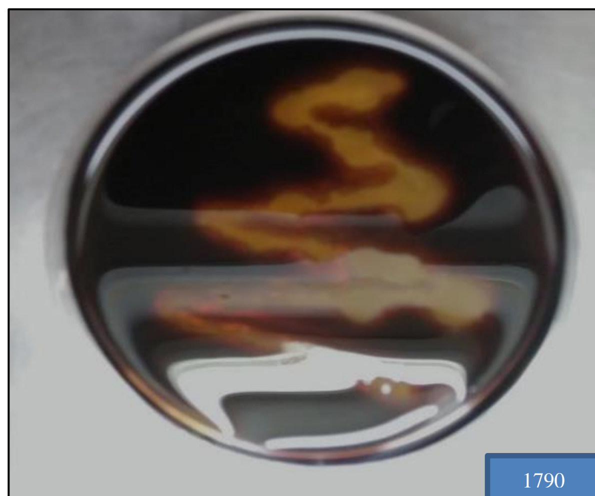
Table 1			
Numbering	**Organims**	**Plate 1**	**Plate 2**
1	**121**	+	+
2	**1790**	++	++
3	**NCDC71**	+++	+++
4	**2941**	**Very low**	**Very low**

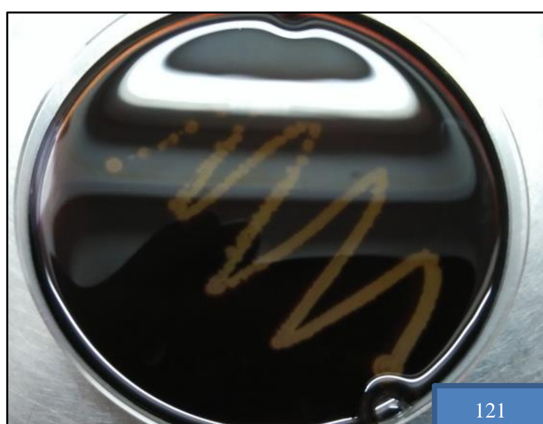
As seen in the table (1) NCDC showed the most activity and larger zone of clearance.



Pic3. Starch agar plates before flooding the plates with Gram Iodine sol^{n}







Pic4. Starch agar plates after flooding the plates with Gram Iodine sol^{n}

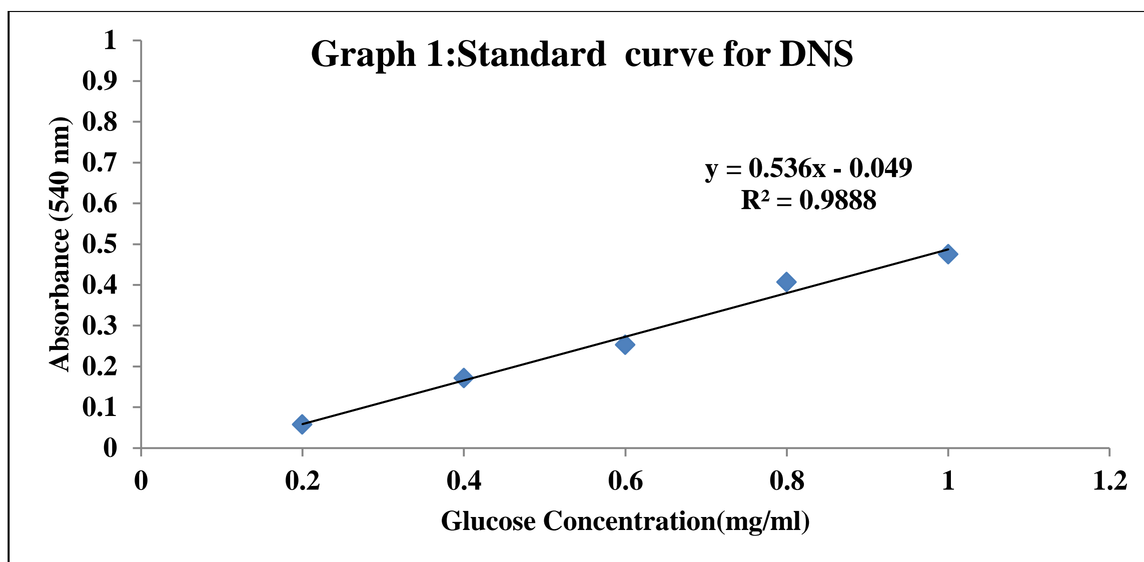
3.3. QUANTITATIVE ANALYSIS OF ENZYME ACTIVITY BY DNS METHOD

Standard Curve: Absorbance was plotted after blank reduction as shown in the table (2).

Table 2		
Tube No.	**Glucose concentration(mg/ml)**	**Absorbance of sample at 540nm**
2	**0.2**	**0.057**
3	**0.4**	**0.171**
4	**0.6**	**0.253**
5	**0.8**	**0.407**
6	**1**	**0.475**

Blank reading: 0.071

Final O.D = O.D of Sample – OD of Blank



Enzyme activity: O.D of samples were taken at 540nm and Enzyme activity was calculated as,

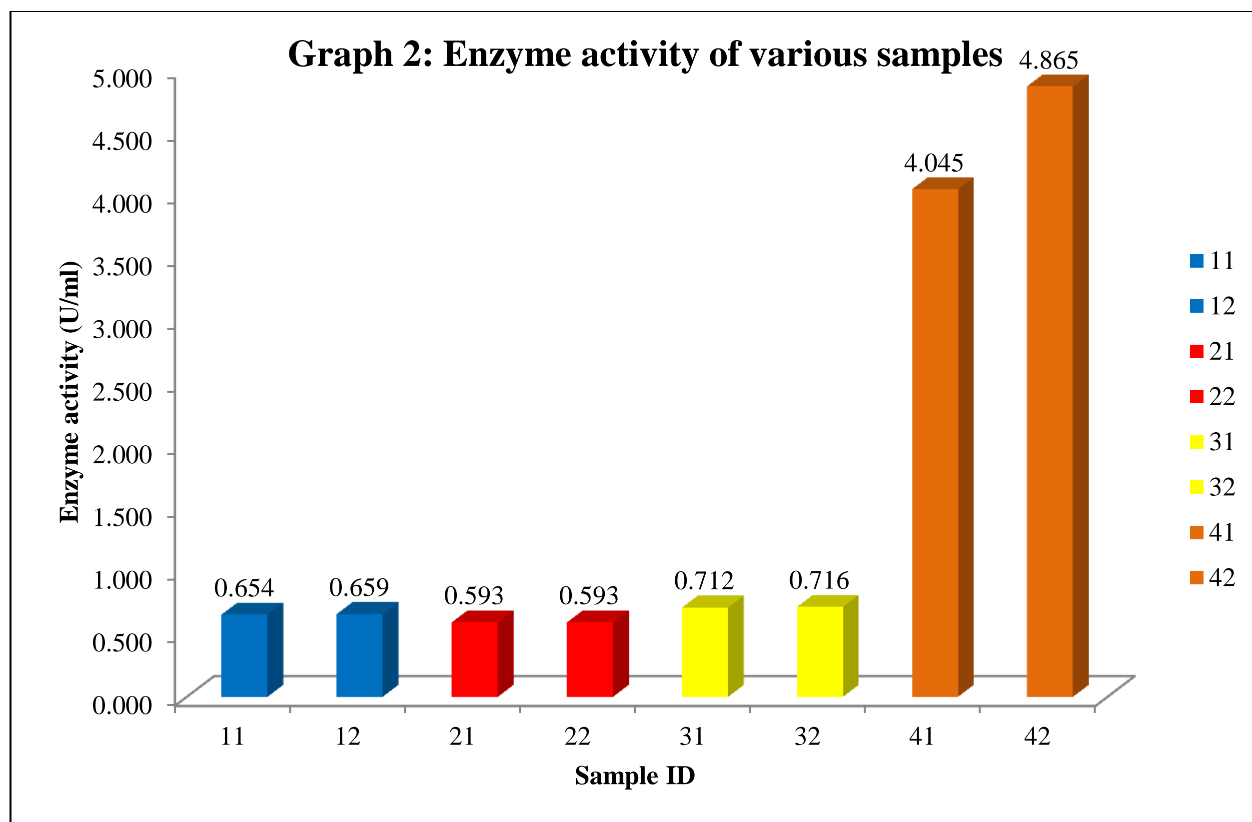
- First Final O.D of the sample was calculated as,
- *Final O.D: O.D of sample – (O.D of Substrate control – O.D of Enzyme Control)*
- Unknown concentration of glucose was calculated from the standard plot, and using that the Enzyme activity was calculated by the formulae,

Enzyme activity (IU/ml) = (Concentration of Glucose (mg/ml)*1000)/ (Incubation time (mins)*Volume of Enzyme (ml)*molecular weight of glucose)

30 degree (Table 3)			
Sample ID	**Absorbance of sample**	**Unknown concentration of Glucose (mg/ml)**	**Enzyme activity(IU/ml)**
11	**0.107**	**0.295**	**0.654**
12	**0.108**	**0.296**	**0.659**
21	**0.092**	**0.267**	**0.593**
22	**0.092**	**0.267**	**0.593**
31	**0.121**	**0.320**	**0.712**
32	**0.122**	**0.322**	**0.716**
41	**0.934**	**1.820**	**4.045**
42	**1.134**	**2.189**	**4.865**

(**Note:** 1,2,3,4 are codes for organisms (121, 1790, NCDC 71, 2941 respectively)

Second digit represents the sample number of that organism, like 11 is the first sample of 121)



Maximum activity was seen in sample 41 and 41 that are of *Lactobacillus plantarum* and then activity seen in bacillus subtilis NCDC 71 is good

Enzyme activity at varying temperatures: Activity of the Enzyme was tested and calculated at various temperatures and O.D taken and calculated as,

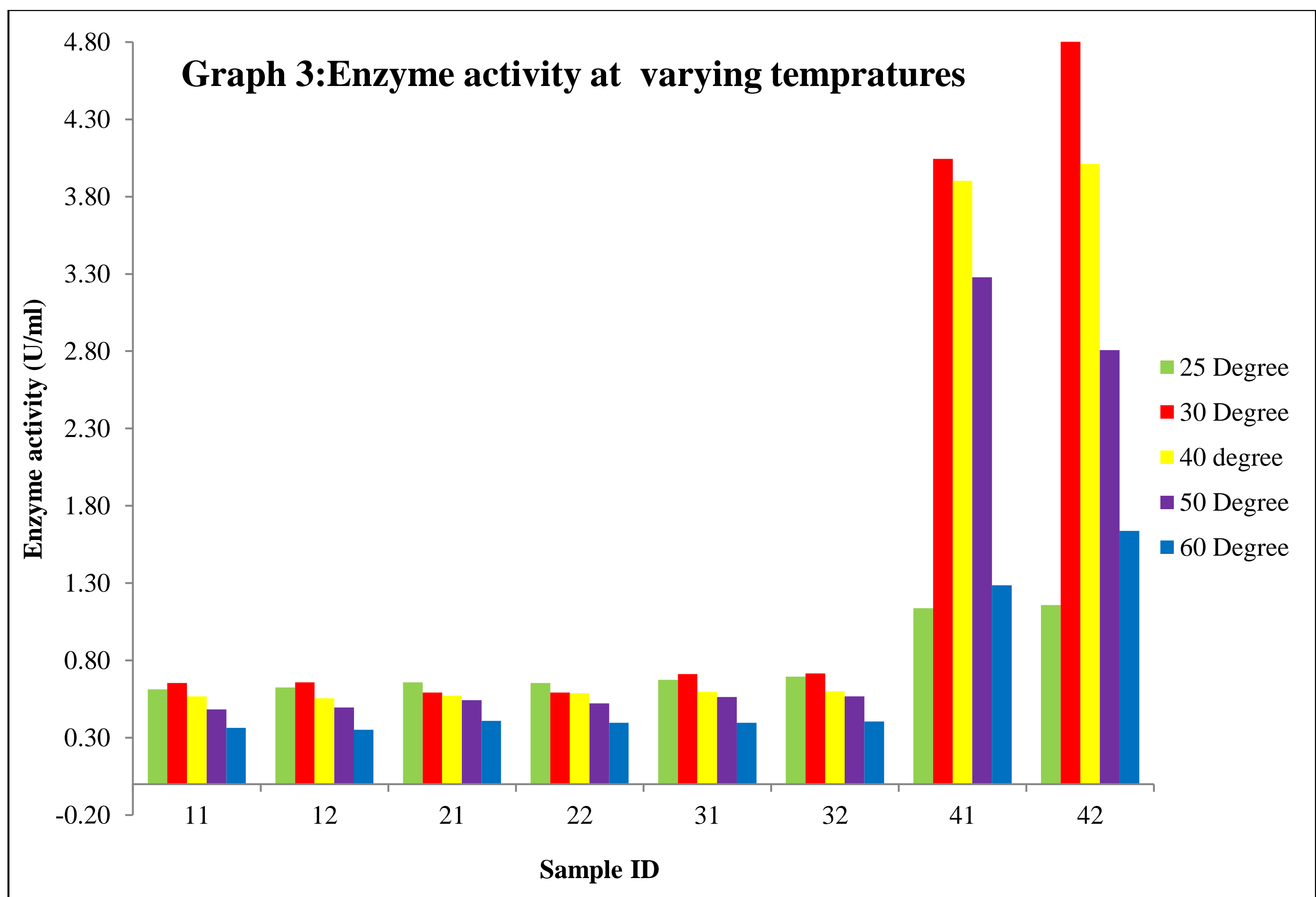
- First Final O.D of the sample was calculated as,
- *Final O.D: O.D of sample – (O.D of Substrate control – O.D of Enzyme Control)*
- Unknown concentration of glucose was calculated from the standard plot, and using that the Enzyme activity was calculated by the formulae,

Enzyme activity (IU/ml) = (Concentration of Glucose (mg/ml)*1000)/ (Incubation time (mins)*Volume of Enzyme (ml)*molecular weight of glucose)

Enzyme activity at various temperature(IU/ml) (Table 4)					
Sample ID	**25**	**30**	**40**	**50**	**60**
11	**0.613**	**0.654**	**0.568**	**0.482**	**0.363**
12	**0.626**	**0.659**	**0.556**	**0.495**	**0.351**
21	**0.659**	**0.593**	**0.572**	**0.544**	**0.408**
22	**0.654**	**0.593**	**0.589**	**0.523**	**0.396**
31	**0.675**	**0.712**	**0.597**	**0.564**	**0.396**
32	**0.695**	**0.716**	**0.601**	**0.568**	**0.404**
41	**1.138**	**4.045**	**3.901**	**3.278**	**1.286**
42	**1.159**	**4.865**	**4.012**	**2.807**	**1.638**

(**Note:** 1,2,3,4 are codes for organisms (121, 1790, NCDC 71, 2941 respectively)

Second digit represents the sample number of that organism, like 11 is the first sample of 121)



Maximum activity of enzymes was seen at 30°C

3.4. PROTEIN PURIFICATION BY AMMONIUM PRECIPITATION

Enzyme activity at 0-30, 30-60, 60-90% concentration: Activity of the Enzyme was tested and calculated at various temperatures and O.D taken and calculated as,

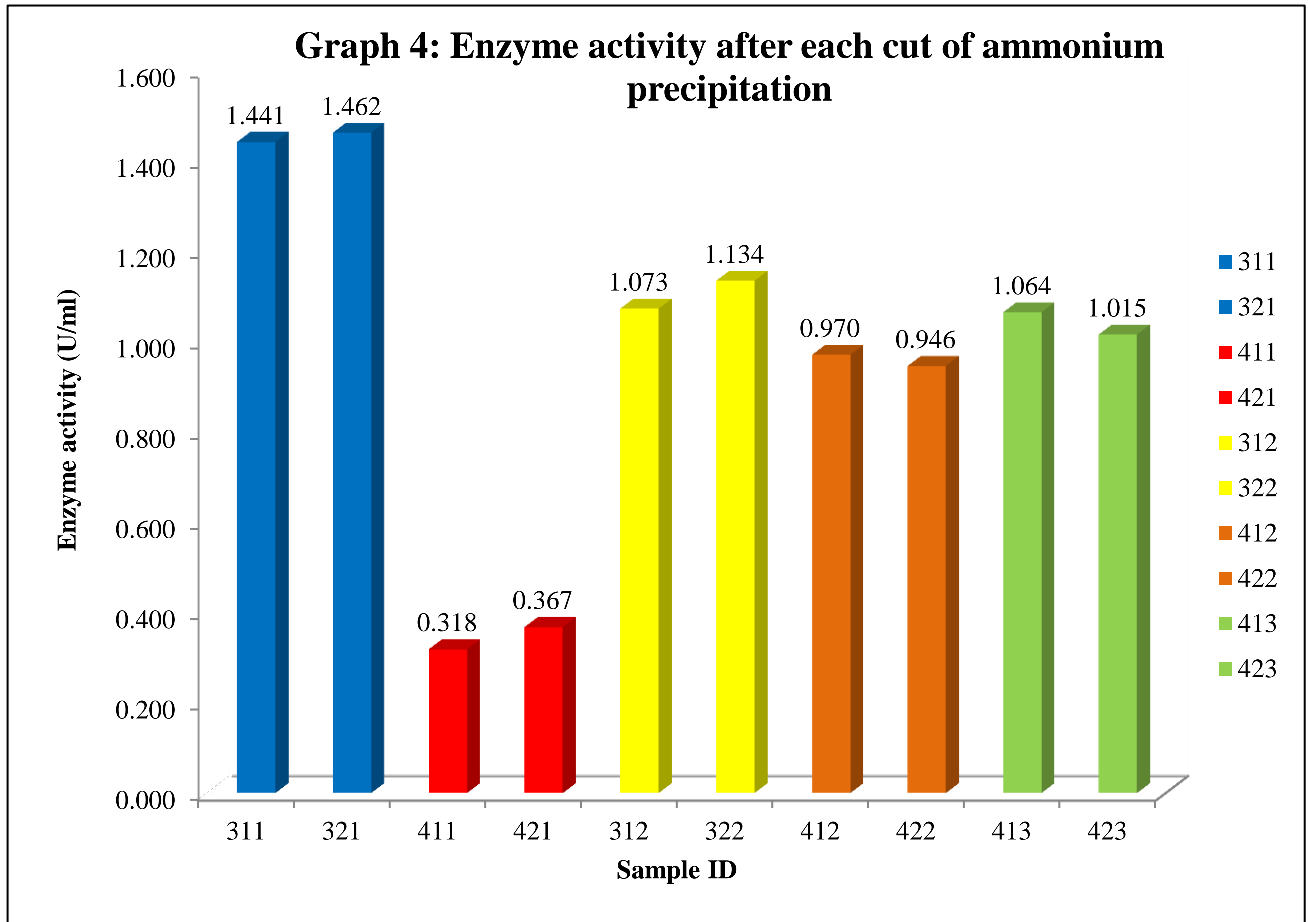
- First Final O.D of the sample was calculated as,
- *Final O.D: O.D of sample – (O.D of Substrate control – O.D of Enzyme Control)*

Table 5			
Sample ID	**Absorbance of sample**	**Unknown concentration of Glucose(mg/ml)**	**Enzyme activity(IU/ml)**
311	0.088	0.259	1.441
321	0.090	0.263	1.462
411	0.025	0.143	0.318
421	0.037	0.165	0.367
312	0.209	0.483	1.073
322	0.224	0.510	1.134
412	0.184	0.437	0.970
422	0.178	0.425	0.946
413	0.207	0.479	1.064
423	0.195	0.457	1.015

(**Note:** 1,2,3,4 are codes for organisms (121, 1790, NCDC 71, 2941 respectively)
Second digit represents the sample number of that organism, like 11 is the first sample of 121
The third digit represents the cut i.e. 0-30% is 1 30-60% is 2 and 60-90% is 3)

- Unknown concentration of glucose was calculated from the standard plot, and using that the Enzyme activity was calculated by the formulae,

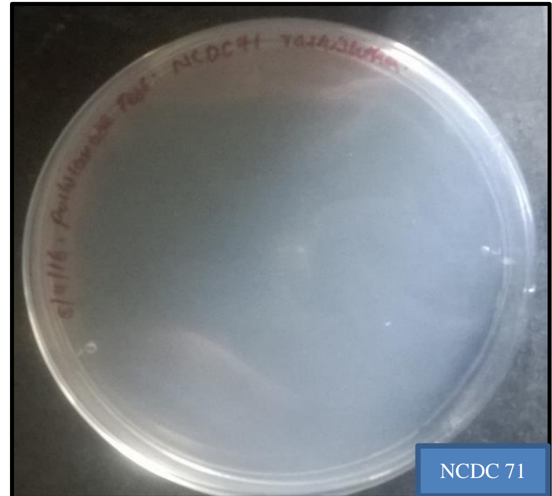
Enzyme activity (IU/ml) = (Concentration of Glucose (mg/ml)*1000)/ (Incubation time (mins)*Volume of Enzyme (ml)*molecular weight of glucose)

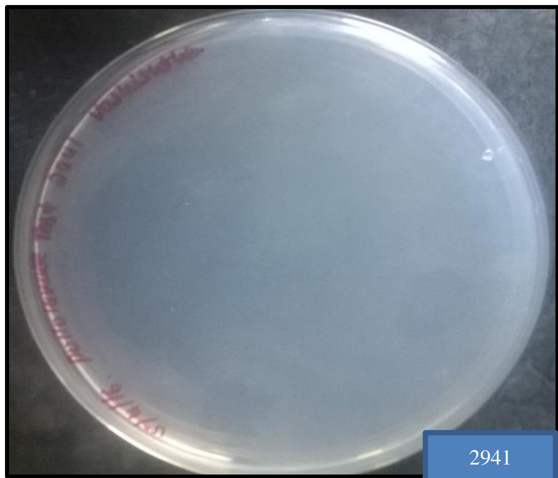


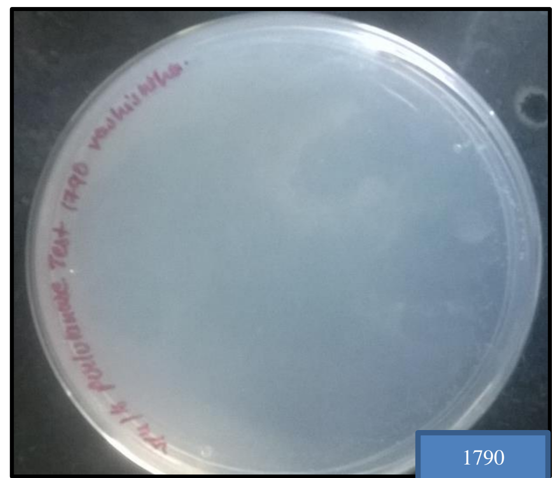
As seen from the table *Bacillus* precipitated maximum at 0-30% cut and the remaining amount at 30-60% cut, for *Lactobacillus* most of the proteins purified at 30-60 and 60-90% cut there was no pellet seen on 60-90% cut in bacillus thus concluding all the proteins purified before 60 % concentration.

3.5. QUALITATIVE ANALYSIS OF PULLULANASE ACTIVITY BY PULLULAN DEGRADING TEST

a. Test 1 with Pullulan agar media composition 1(annexure 9)





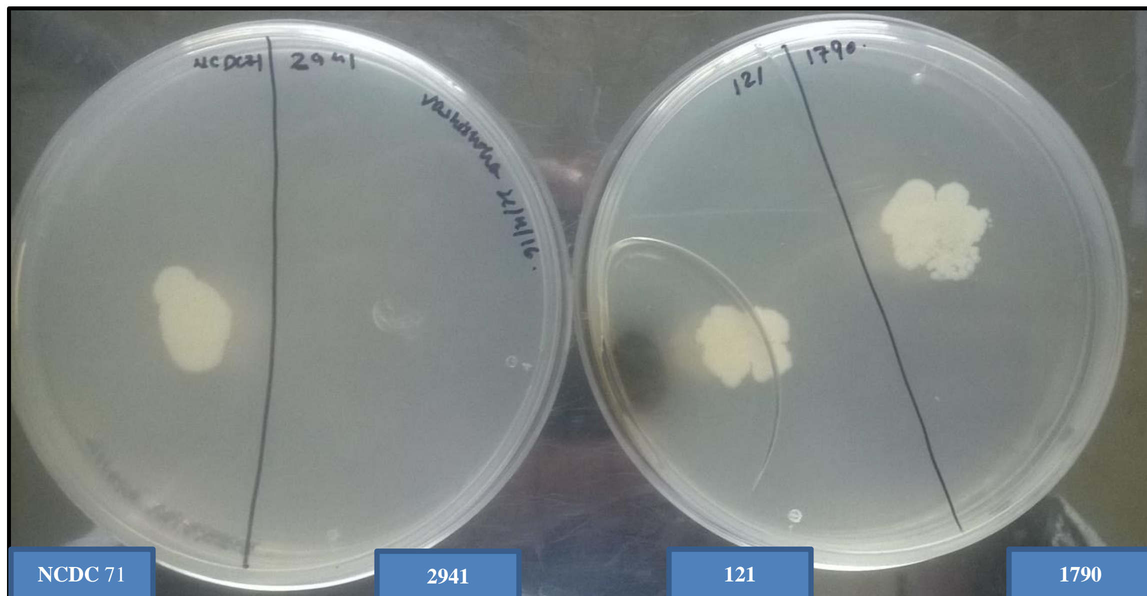


Pic5. Pullulan agar plates after culturing for test 1

Table 6			
Numbering	**Organims**	**Plate 1**	**Plate 2**
1	121	No Growth	No Growth
2	1790	No Growth	No Growth
3	NCDC71	No Growth	No Growth
4	2941	No Growth	No Growth

- No growth was seen in any of the cultures grown for the pullulanase test.
- Possibilities for negative results can be, media not suitable for the growth of the cultures or the substrate (pullulan) used was of old batch and may have degraded.
- Another experiment was performed using the different and simpler media i.e. the pullulan agar media comprising of beef extract and pullulan only and with new batch of pullulan substrate used.

b. Test 2 with Pullulan agar media composition 2(annexure 10)



Pic6. Pullulan agar plates after culturing for test 2

- Growth was seen in the plates with different composition of media except in 2941 *Lactobacillus* plate. As shown in the table below.

- Zone of clearance was slightly observed with colorless zone which showed a very low activity of the enzymes in respective cultures, confirmation of which was done by quantitative assay.

Table 7			
Numbering	**Organims**	**Plate 1**	**Plate 2**
1	**121**	+	+
2	**1790**	+	+
3	**NCDC71**	+	+
4	**2941**	**No growth**	**No growth**

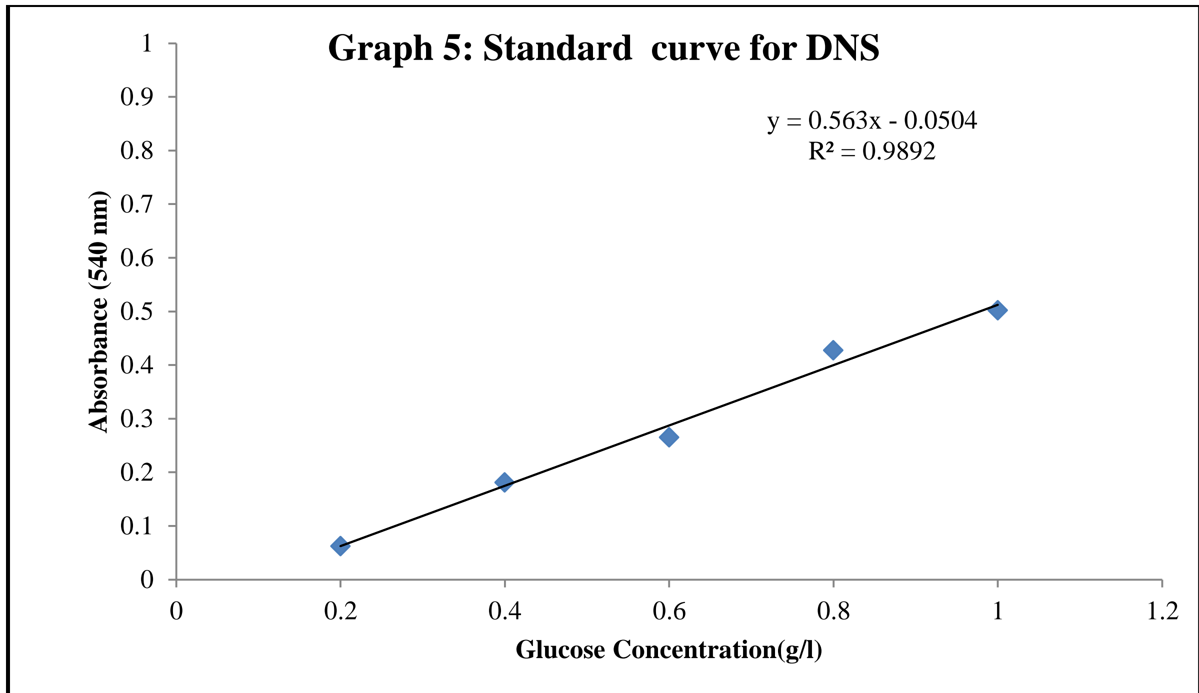
3.6.QUANTITATIVE ANALYSIS OF PULLULANASE ACTIVITY BY DNS METHOD

Standard Curve: Absorbance was plotted after blank reduction as shown in the table.

Table 8		
Tube	**Glucose concentration(g/L)**	**Absorbance of Sample at 540 nm**
2	**0.2**	**0.062**
3	**0.4**	**0.181**
4	**0.6**	**0.265**
5	**0.8**	**0.427**
6	**1**	**0.502**

Tube 1 Blank reading: 0.071

Final O.D = O.D of Sample – OD of Blank



a. Activity assay at temperature 50^{0}C

Enzyme activity: O.D of samples were taken at 540nm and Enzyme activity was calculated as,

- First Final O.D of the sample was calculated as,
- *Final O.D: O.D of sample – (O.D of Substrate control – O.D of Enzyme Control)*
- Unknown concentration of glucose was calculated from the standard plot, and using that the Enzyme activity was calculated by the formulae,

Enzyme activity (IU/ml) = (Concentration of Glucose (mg/ml)*1000)/ (Incubation time (mins)*Volume of Enzyme (ml)*molecular weight of glucose)

No activity was seen in the samples

b. Activity assay after Ammonium precipitation

- No activity was seen in the above sample before and after ammonium precipitation.
- Ammonium sulfate interferes with the enzyme activity assay.
- Possible reason for negative readings might be the temperature at which activity was taken, as organisms grow at 37° C and enzyme would have degraded at 50° C.

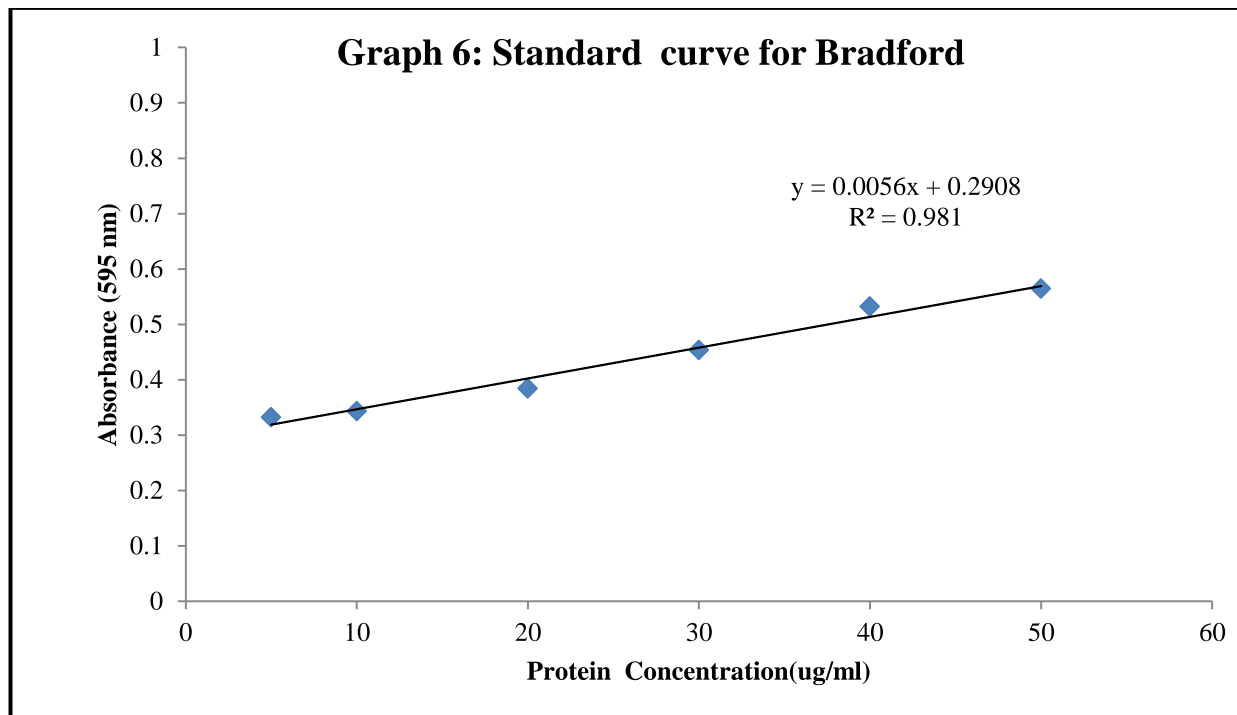
3.7.PROTEIN ESTIMATION USING BRADFORD METHOD

Standard Curve: Absorbance was plotted after blank reduction as shown in the table. Sample OD was taken in triplets and the average of all was calculated respectively and used.

Table 9		
Tube	**BSA protein concentration (ug/ml)**	**Absorbance at 595 nm**
1	**5**	**0.332**
2	**10**	**0.343**
3	**20**	**0.384**
4	**30**	**0.453**
5	**40**	**0.532**
6	**50**	**0.564**

Blank tube: 0.383

Final O.D = O.D of Sample – OD of Blank

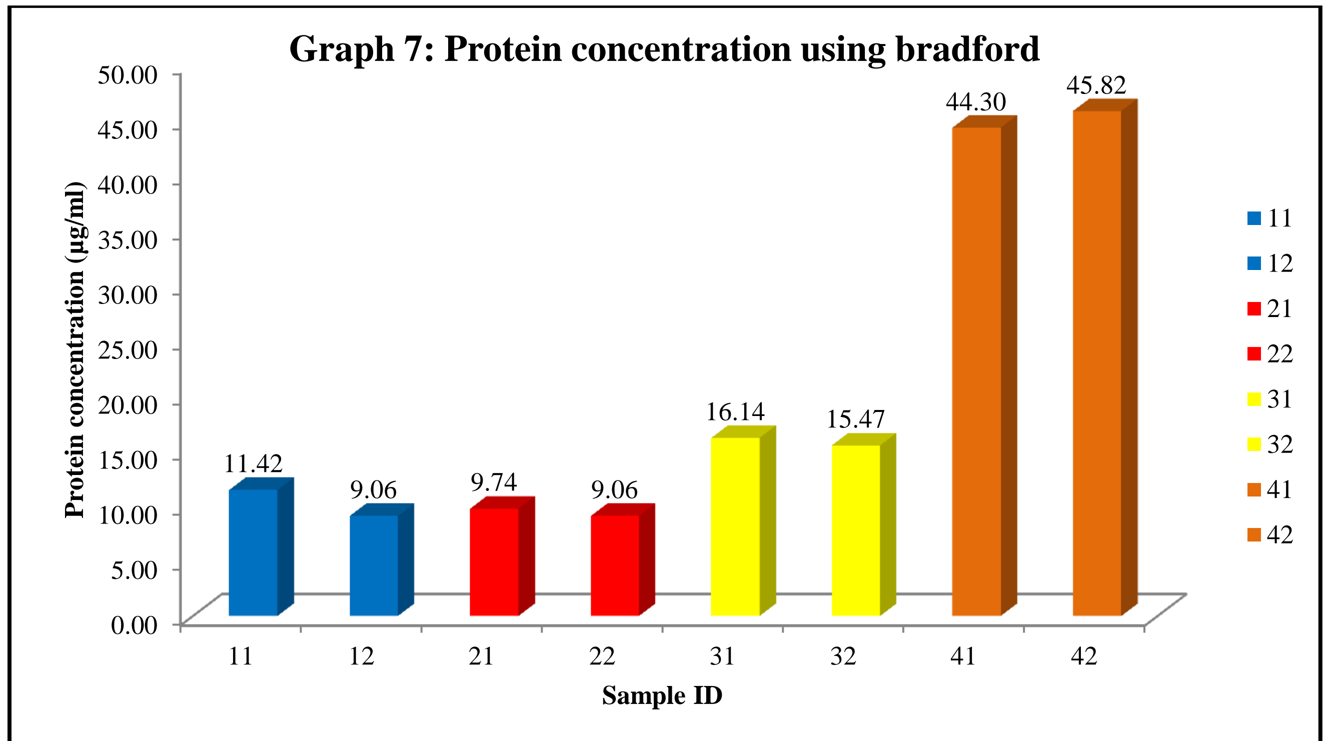


Enzyme concentration:

- O.D of samples was taken at 595nm.
- Final O.D of the sample was calculated from the graph.

Table 10		
Sample code	Absorbance of the enzyme	Unknown concentration (ug/ml)
11	0.352	11.42
12	0.338	9.06
21	0.342	9.74
22	0.338	9.06
31	0.38	16.14
32	0.376	15.47
41	0.547	44.30
42	0.556	45.82

(**Note:** 1,2,3,4 are codes for organisms (121, 1790, NCDC 71, 2941 respectively)
Second digit represents the sample number of that organism, like 11 is the first sample of 121)



Maximum concentration of protein was seen in sample 41 and 41 that are of *Lactobacillus plantarum* and then in *bacillus subtilis* NCDC 71 is good.

3.8. COMPARATIVE ACTIVITY ANALYSIS OF BOTH THE ENZYMES

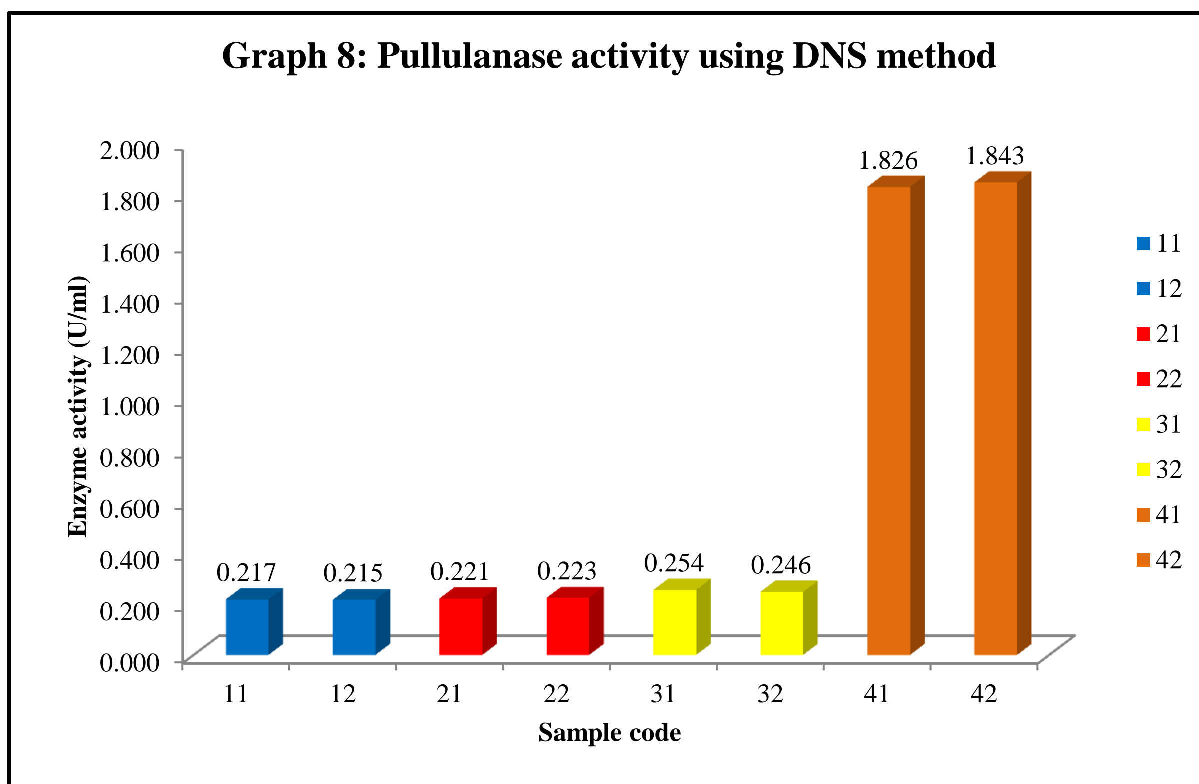
Enzyme activity: O.D of samples were taken at 540nm and Enzyme activity was calculated as,

- First Final O.D of the sample was calculated as,
- **Final O.D:** O.D of sample – (O.D of Substrate control – O.D of Enzyme Control)
- Unknown concentration of glucose was calculated from the standard plot, and using that the Enzyme activity was calculated by the formulae,

Enzyme activity (IU/ml) = (Concentration of Glucose (mg/ml)*1000)/ (Incubation time (mins)*Volume of Enzyme (ml)*molecular weight of glucose)

Pullulanase activity (Table 11)			
Sample code	**Absorbance of sample**	**Unknown concentration (mg/ml)**	**Enzyme activity(IU/ml)**
11	**0.057**	**0.195**	**0.217**
12	**0.056**	**0.193**	**0.215**
21	**0.059**	**0.199**	**0.221**
22	**0.06**	**0.200**	**0.223**
31	**0.076**	**0.229**	**0.254**
32	**0.072**	**0.222**	**0.246**
41	**0.881**	**1.643**	**1.826**
42	**0.89**	**1.659**	**1.843**

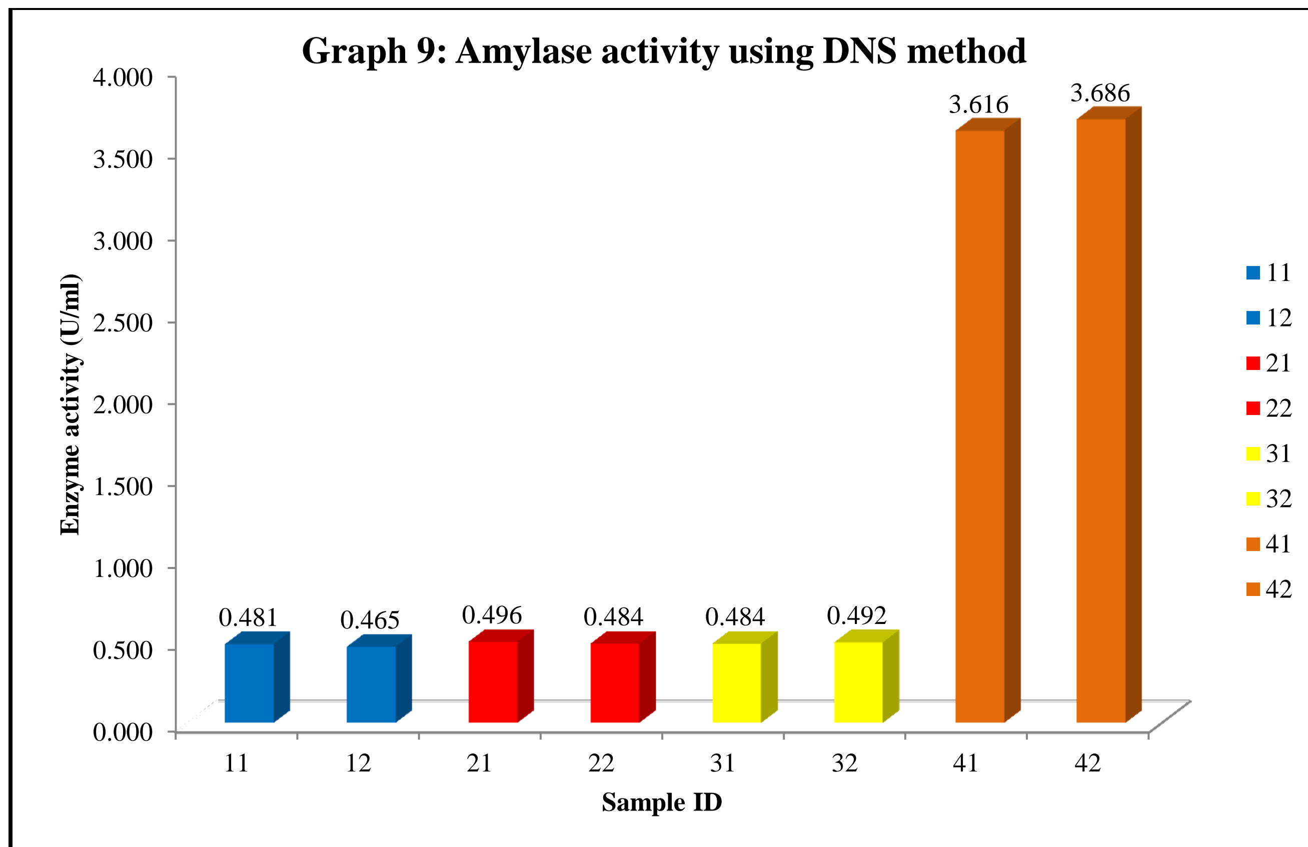
(**Note:** 1,2,3,4 are codes for organisms (121, 1790, NCDC 71, 2941 respectively)
Second digit represents the sample number of that organism, like 11 is the first sample of 121)



- Pullulanase activity was seen this time at 30° C.

Amylase activity (Table 12)			
Sample ID	**Absorbance of sample**	**Unknown concentration (mg/ml)**	**Enzyme activity(IU/ml)**
11	**0.069**	**0.216**	**0.481**
12	**0.065**	**0.209**	**0.465**
21	**0.073**	**0.223**	**0.496**
22	**0.07**	**0.218**	**0.484**
31	**0.07**	**0.218**	**0.484**
32	**0.072**	**0.222**	**0.492**
41	**0.872**	**1.627**	**3.616**
42	**0.89**	**1.659**	**3.686**

(**Note:** 1,2,3,4 are codes for organisms (121, 1790, NCDC 71, 2941 respectively)
Second digit represents the sample number of that organism, like 11 is the first sample of 121)

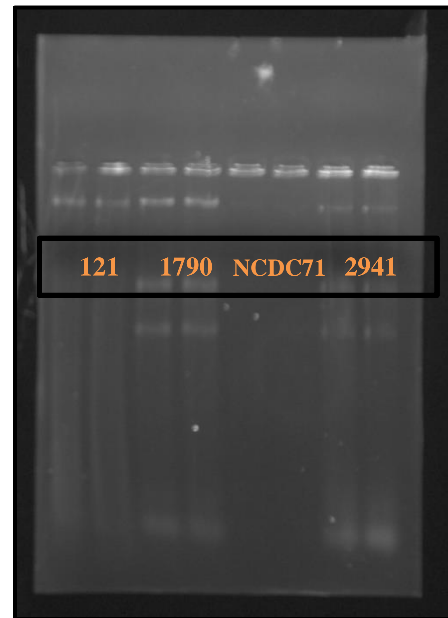


- As seen in the above graphs for pullulanase and amylase activity organisms showed good amylase activity as compared to pullulanase activity.
- Lactobacillus plantarum showed high activity for both the enzymes amylase and pullulanase followed by NCDC 71.

3.9. DNA ISOLATION

DNA was visualized using Gel Doc system and image was taken as shown in pic 4, DNA was seen in all samples except NCDC 71 due to low concentration of isolated DNA or may be due to some manual error.

Readings were also taken using Nanodrop spectrophotometer at 260 nm.



Pic 7 Gel image for isolated DNA

Table 13	
Sample ID	**DNA Concentration(ng/µl)**
11	94.9
12	92
21	89.1
22	82.1
31	57.1
32	51.1
41	206
42	211

(**Note:** 1,2,3,4 are codes for organisms (121, 1790, NCDC 71, 2941 respectively)
Second digit represents the sample number of that organism, like 11 is the first sample of 121)

Formulae to calculate DNA concentration= 50 µg/mL × O.D 260 × dilution factor

Readings of DNA at 260/280 absorbance for purity were 1.8, 1.5, 1.7, 1.88 respectively thus, the DNA isolated was pure free from any protein contamination or RNA contamination. But the Absorbance at 260/230 was low compared to the accepted units of 2-2.2; hence DNA had contamination of other substances like phenols etc.

3.10. Acquiring Protein sequence and structure.

- Sequences were acquired from NCBI and structures from PDB, details of which are shown in table 12.

Table 14

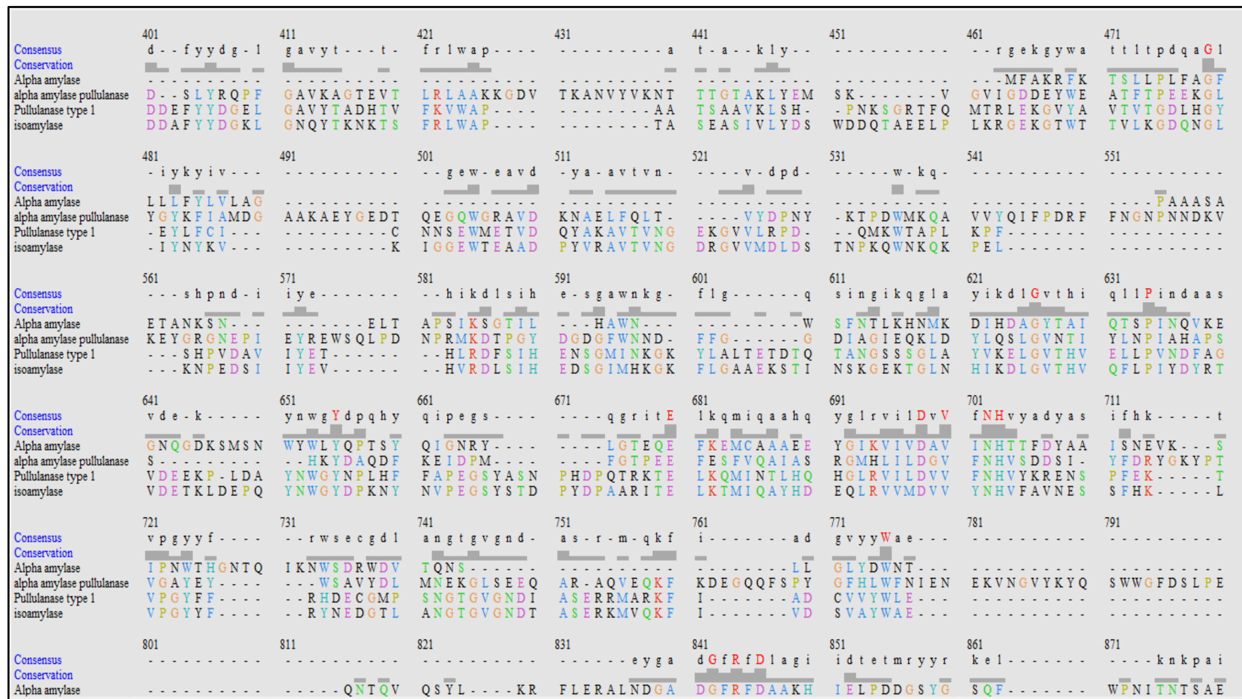
Name	Organism	Accession number	Length	Bonds processed	PDB ID
Pullulanase Type I	***Bacillus subtilis***	**255767686**	**718**	**1,6**	**2E8Y**
Pullulanase Type II	***Bacillus subtilis***	**460686**	**2032**	**1,6 & 1,4**	**-**
Isoamylase	***Bacillus lentus***	**493116169**	**886**	**1,6**	**-**
Alpha amylase	***Bacillus subtilis***	**142435**	**425**	**1,4**	**1BAG**

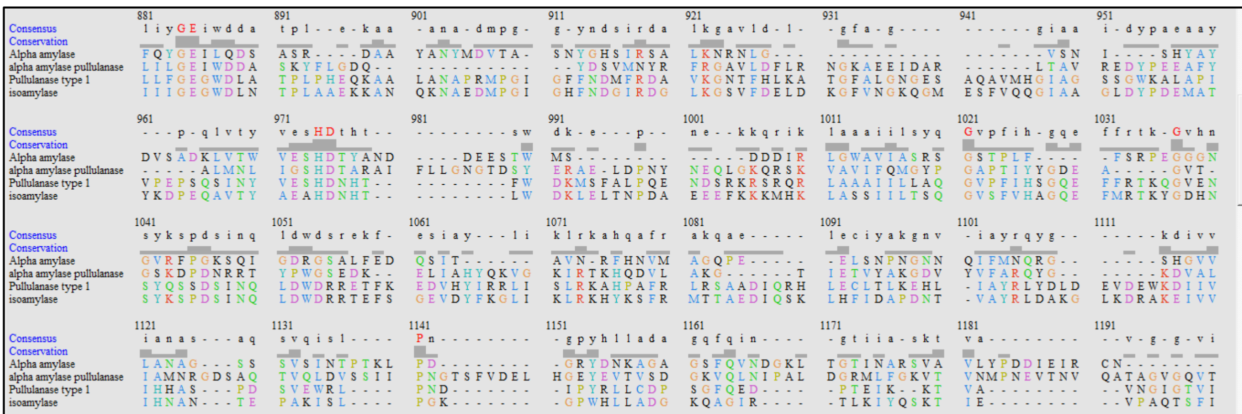
3.11. Multiple sequence alignment using Clustalw

- Multiple sequence alignment of the sequences were done and results were shown as below along with the identity matrix:

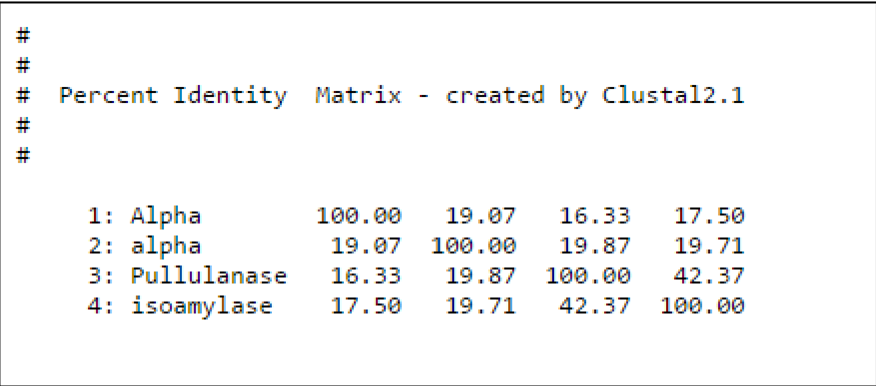
```
                          1          11         21         31         41         51         61         71
Consensus                 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Conservation
Alpha amylase             ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
alpha amylase pullulanase MVKGRWRKYV AYFSIFLLFA QLFSFSAIVR ANEPMQSPVV NGNDVTFRYV GTGEEQSVLL AGSFNDWQTI ELTKESDYIW
Pullulanase type 1        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
isoamylase                ---------- ---------- ---------- --------MV QGIH------ ---------- ---------- ---DEVGFI-

                          81         91         101        111        121        131        141        151
Consensus                 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Conservation
Alpha amylase             ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
alpha amylase pullulanase SVTKTLPDGT YMYKFVVDGN WVADPLNANQ ADDGHGGKNS VVVVGQPAQQ QERVVTLVGN LQKELGHEKE WDPTATATVM
Pullulanase type 1        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
isoamylase                ---------- -----VRTDS WEKD------ -----GGDRF I--------- ---------- -NVQSGNDEV WVKAGDEHTY

                          161        171        181        191        201        211        221        231
Consensus                 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Conservation
Alpha amylase             ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
alpha amylase pullulanase KHEGNGLYTF TGTL-----P AGTYEYKIAI NGSWDENYGA GGRNGGNISF TLEKETEV-- ---------- ----------
Pullulanase type 1        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---MVSIRR-
isoamylase                TSPPDGEYKD FPAFERVKVK LHYFRYDGN- YEGWNLWTWP EGKDGKRVDF TAEDDFGKVA EFELENQEGM KRAGFIVRKS

                          241        251        261        271        281        291        301        311
Consensus                 ---------- -------y-d d-------i- a---si---- ---------v ----p-f--e te-t-fp--- --------r-
Conservation
Alpha amylase             ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
alpha amylase pullulanase ---------- -----TFYYH DS------TH AIADSTWYTP IPKEKQPRLV GTILPAIGYE KEVDGWTPET STAFFTDDDF
Pullulanase type 1        ---------- ---SFEAYVD DMNIITVLIP AEQKEI---- ---------M ---TPPFRLE TEITDFPL-- -----AVR--
isoamylase                MEGNDWAGRE FGDRFITKFD ENGNAEIWIV QGTESIYYNP DYIEKDPKIV NASMDTFNQI TLETNFPF-- -----QWRDW

                          321        331        341        351        361        371        381        391
Consensus                 ---------- ---------- ------eey- --n--k--vv -e---tf--- -k-------- a-sg----v- ig---r---f
Conservation
Alpha amylase             ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
alpha amylase pullulanase DSVYTFSARV PKGTYEYKVV LGNSWDENYP QDNNAKLNVL EETTITFFFN AKTKELYTDY SPTGSDGNVD KD-RLKHNTW
Pullulanase type 1        ---------- ---------- ------EEYS LEAKYKYVCV SDHPVTFGKI HCVR------ ASSGHKTDLQ IGAVIRTAAF
isoamylase                KSIINIDN-- ----ADIKEV VPYDGNEESN VTN--KVKVV TEDKLDFRQS YKIS------ AVSFGEAEVK IGEIVRSKEF

                          401        411        421        431        441        451        461        471
Consensus                 d--fyydg-l gavyt---t- frlwap---- ---------a t-a--kly-- ---------- --rgekgywa ttltpdqaGl
Conservation
Alpha amylase             ---------- ---------- ---------- ---------- ---------- ---------- ---MFAKRFK TSLLPLFAGF
alpha amylase pullulanase D--SLYRQPF GAVKAGTEVT LRLAAKKGDV TKANVYVKNT TTGTAKLYEM SK-------V GVIGDDEYWE ATFTPEEKGL
```

Pic 8: Multiple alignments.





Pic 9, 10: Multiple alignments.



#

#

Percent Identity Matrix - created by Clustal2.1

#

#

1: Alpha	100.00	19.07	16.33	17.50
2: alpha	19.07	100.00	19.87	19.71
3: Pullulanase	16.33	19.87	100.00	42.37
4: isoamylase	17.50	19.71	42.37	100.00

Pic 11: Identity Matrix

```
                           1201        1211        1221        1231        1241        1251        1261        1271
Consensus                  1---------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
Conservation
Alpha amylase              ----------  TFFQ------  ----------  ----------  ----------  ----------  ----------  ----------
alpha amylase pullulanase  LAWEGDASTY  RIYQSTLKGA  GYELVKETNE  KTAVIDNLKN  GTAYYFAITA  VDENGNESAK  VETNRVVPHY  PLAEEHVALL
Pullulanase type 1         LYLASDLKSF  A---------  ----------  ----------  ----------  ----------  ----------  ----------
isoamylase                 LKR-------  ----------  ----------  ----------  ----------  ----------  ----------  ----------

                           1281        1291        1301        1311        1321        1331        1341        1351
Consensus                  ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
Conservation
Alpha amylase              ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
alpha amylase pullulanase  SEVNGGVLDL  AALITVEAKV  QIDNVTKHGL  ADGLQAVLQV  KKPNSDKWED  IQATYDRQDG  DANVFRASFT  PLQAGTYTYR
Pullulanase type 1         ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
isoamylase                 ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------

                           1361        1371        1381        1391        1401        1411        1421        1431
Consensus                  ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
Conservation
Alpha amylase              ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
alpha amylase pullulanase  YGFTTNLGDS  WVYTEEKTFT  LTANEADQQA  PAKGIHLLQP  DVESGQVNLS  WSFIERDDHD  AYMVVIERDG  QVIHTTTTIS
Pullulanase type 1         ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
isoamylase                 ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------

                           1441        1451        1461        1471        1481        1491        1501        1511
Consensus                  ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
Conservation
Alpha amylase              ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
alpha amylase pullulanase  TSFTDYDVEN  GKTYTYVVKL  YDRAGNVVAS  KDVQITPDIV  MVQVTFKVKA  PSYTPLDTRI  TIPNSINGWN  TGAWEMTRGG
Pullulanase type 1         ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
isoamylase                 ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------

                           1521        1531        1541        1551        1561        1571        1581        1591
Consensus                  ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
Conservation
Alpha amylase              ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
alpha amylase pullulanase  AVTPDWEFTT  ELQEGETITY  KYVKGGSWDQ  EGLADHTRDD  QTDDDVSYYG  YGAIGTELKV  TVHNQGNNKM  VIQDYILRWI
Pullulanase type 1         ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
isoamylase                 ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------

                           1601        1611        1621        1631        1641        1651        1661        1671
Consensus                  ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
Conservation
Alpha amylase              ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
```

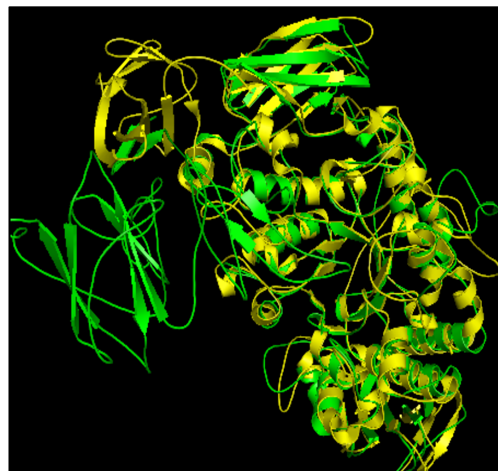
Pic 12: Multiple alignments.

- Very low Identity was seen in the sequences for the sequence alignment.
- A satisfactory match for the sequence of isoamylase and Pullulanase type I was seen.

3.12. Structure alignment of the Proteins.

a. Alignment of whole protein structure:

Pic 13: Structural alignment Pullulanase type I vs Alpha amylase



Pic 14: Structural alignment Pullulanase type I vs Isoamylase

- Very low similarity was seen when proteins were aligned as a whole.
- Isoamylase and pullulanase type I showed good similarity.

b. Domain analysis:

- Various domains present in the proteins whick are responsible for the activity are given below in table 12:

Table 15			
Enzyme	**Conserved Domain**	**Role**	**Interval**
Pullulanase type I	AmyAc_Pullulanase_LD-like	Alpha amylase catalytic domain found in pullulanase	213-613
Pullulanse type II	AmyAc_CMD	Alpha amylase catalytic domain, cleaves 1, 4 and 1, 6 bond.	475-952
Isoamylase	AmyAc_Pullulanase_LD-like	Alpha amylase catalytic domain found in pullulanase	386-787
Alpha amylase	AmyAc_bac1_AmyA	Alpha amylase catalytic domain found in bacterial Alpha-amylases	50-393

3.13. Conclusion

All the objectives of the study were completed. Production of starch debranching enzymes was done. Amylase showed good activity of 4.8 U/ml in *Lactobacillus plantarum* and satisfactory in *Bacillus subtilis* NCDC 71. Enzyme activity was best seen at temperature of 30 degree at pH 7.Ammonium precipitation showed that the strains of Bacillus 121, 1790, NCDC 71 were completely precipitated till concentration of 60% and 2941 till 90%.

Pullulanase activity was also seen in the bacterial strains. Activity was seen at temperature of 30 degree and was low as compared to amylase activity. Both the enzymes produced were extracellular.

Alignment of the sequence and structure showed unpromising results, with very low sequence identity but when the domains were analyzed all enzymes had common active site of D (Aspartic acid), E (Glutamic acid), D and showed 50 percent catalytic site similarity.

So for further studies alignment can be performed to see the similarity in the active domains and their catalytic sites to decipher the relation in between these enzymes

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4.2. ANNEXURE

1. LB media composition

Composition	Quantity
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Distilled water	1 L
pH	7.0

2. LMRS media composition

Composition	Quantity
Protease peptone	10 g
Beef extract	10 g
Yeast extract	5 g
Dextrose	20 g
Polysorbate 80	1 g
Ammonium citrate	2 g
Sodium acetate	5 g
Magnesium sulphate	0.1 g
Manganese sulphate	0.05 g
Di-potassium phosphate	2
Distilled water	1 L
pH	6.5±0.2

3. Starch agar media composition

Composition	Quantity
Beef extract	3g
Soluble starch	10g
Agar	12g
Distilled water	1 L

4. **DNS Reagent composition**

Composition	Quantity
DNS reagent	1g
2 M Sodium hydroxide(NaOH)	20 ml
Sodium potassium tartrate	30 g
Distilled water	80 ml (approx.)

Made by adding 1 g of DNS in 20 ml 2M NaOH, add water to make volume of 60 ml and slowly add sodium potassium tartrate 30 g by heating and continuous mixing on hot plate magnetic stirrer, and dilute to final volume of 100 ml.

5. **Sodium phosphate buffer**

Composition	Concentration Needed	Added to final Buffer
Mono basic Dihydrogen Phosphate (1)	1 M	
Dibasic Mono Hydrogen Phosphate(2)	1 M	
Sodium Phosphate Buffer	1 M (*48 ml (1) +52 ml (2)*)	
Sodium Chloride (NaCl)	1 M	
Sodium phosphate concentration for final buffer	**0.02**	**2 ml**
NaCl concentration for final buffer	**0.006**	**0.6 ml**
Distilled water		**97.4 ml**

Above table shows the stepwise approach of making final buffer solution.

6. **DNA Isolation buffers**
TNE Buffer:

Composition	Concentration
Tris HCl (pH 7.4)	50mM
NaCl	100mM
EDTA	0.1mM

SET Buffer/Lysis Buffer

Composition	Concentration
Tris HCl (pH 8)	100mM
SDS	1 % w/v
EDTA	50mM

T.E Buffer

Composition	Concentration
Tris HCl (pH 8)	10mM
EDTA	0.5M

7. **Buffer required for gel Run**
   SB buffer:

Composition	Quantity Stock (20X)
Boric acid	45 g
NaOH	8 g

SB Buffer working solution (1X can be made from 20X stock solution)

Loading Dye:

Composition	Concentration
Glycerol (30%)	3ml
Bromophenol blue (0.25%)	25 mg
dH_2O	10 ml

8. **Ammonium sulfate precipitation chart**

Initial concentration of ammonium sulphate, % saturation	Final concentration of ammonium sulphate—% saturation at 0 °C																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	g solid ammonium sulphate to add to 100 ml of solution																
0	10·6	13·4	16·4	19·4	22·6	25·8	29·1	32·6	36·1	39·8	43·6	47·6	51·6	55·9	60·3	65·0	69·7
5	7·9	10·8	13·7	16·6	19·7	22·9	26·2	29·6	33·1	36·8	40·5	44·4	48·4	52·6	57·0	61·5	66·2
10	5·3	8·1	10·9	13·9	16·9	20·0	23·3	26·6	30·1	33·7	37·4	41·2	45·2	49·3	53·6	58·1	62·7
15	2·6	5·4	8·2	11·1	14·1	17·2	20·4	23·7	27·1	30·6	34·3	38·1	42·0	46·0	50·3	54·7	59·2
20	0	2·7	5·5	8·3	11·3	14·3	17·5	20·7	24·1	27·6	31·2	34·9	38·7	42·7	46·9	51·2	55·7
25		0	2·7	5·6	8·4	11·5	14·6	17·9	21·1	24·5	28·0	31·7	35·5	39·5	43·6	47·8	52·2
30			0	2·8	5·6	8·6	11·7	14·8	18·1	21·4	24·9	28·5	32·3	36·2	40·2	44·5	48·8
35				0	2·8	5·7	8·7	11·8	15·1	18·4	21·8	25·4	29·1	32·9	36·9	41·0	45·3
40					0	2·9	5·8	8·9	12·0	15·3	18·7	22·2	25·8	29·6	33·5	37·6	41·8
45						0	2·9	5·9	9·0	12·3	15·6	19·0	22·6	26·3	30·2	34·2	38·3
50							0	3·0	6·0	9·2	12·5	15·9	19·4	23·0	26·8	30·8	34·8
55								0	3·0	6·1	9·3	12·7	16·1	19·7	23·5	27·3	31·3
60									0	3·1	6·2	9·5	12·9	16·4	20·1	23·9	27·9
65										0	3·1	6·3	9·7	13·2	16·8	20·5	24·4
70											0	3·2	6·5	9·9	13·4	17·1	20·9
75												0	3·2	6·6	10·1	13·7	17·4
80													0	3·3	6·7	10·3	13·9
85														0	3·4	6·8	10·5
90															0	3·4	7·0
95																0	3·5
100																	0

9. **Pullulan agar media composition 1**

Composition	Quantity(g/l)
Pullulan	10
NaCl	2
$MgSO_4.7H_2O$	0.1
K_2HPO_4	0.17
$KH_2PO_4.7H_2O$	0.12
Agar	15
pH	7.5±0.2

10. **Pullulan agar media composition 2**

Composition	Quantity
Beef extract	3g
Pullulan	10g
Agar	12g
Distilled water	1 L